THE ROLE OF P73 IN CANCER – A PATHWAY TOWARDS MORE EFFECTIVE CANCER THERAPY

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Tiivistelmä – Referat – Abstract

The p53-family consists of three transcription factors, p53, p73 and p63. The family members have similar but also individual functions connected to cell cycle regulation, development and tumorigenesis. p53 and p73 act mainly as tumor suppressors. During DNA damage caused by anticancer drugs or irradiation, p53 and p73 levels are upregulated in cancer cells leading to apoptosis and cell cycle arrest. p53 is mutated in almost 50 per cent of the cancers, causing the cancer cells unable to undergo cell death. Instead, p73 is rarely mutated in cancer cells and because of that could be more viable target for anticancer therapy. The network surrounding the regulation of p73 is extensive and has several potential targets for cancer therapy. One of the most studied is Itch ligase, the negative regulator of p73 levels. Gene therapy directed towards knockdown of Itch ligase is a potential approach but in need for more in vivo proof. p73 has two isoforms, transactivating TA-forms and dominant-negative ΔN-forms. The specific regulation of these isoforms could also offer a possible way for more effective cancer treatment.

The literature work includes information of structures, isoforms, functions and possible therapeutic targets of p73. Also the main therapeutic approaches to date are introduced. The experimental part is based on transfection and cytotoxicity studies done e.g. in pancreatic cancer cells (Mia PaCa-2, PANC1, BxPc-3 and HPAC). The aim of the experimental work was to optimize the conditions for effective transfection with DAB16 dendrimer nanoparticles and to measure the cytotoxicity of plain dendrimers and DAB16-pDNA complexes. Also the protein levels of p73 and Itch ligase were measured by Western blotting. The work was done as a part of a bigger project, which was aiming to down regulate Itch ligase (negative regulator of p73) by siRNA/shRNA. Tranfection results were promising, showing good transfection efficacy with DAB16 N/P30 in pancreatic cancer cells (except in BxPc-3). Pancreatic cancer cells showed recovery in 3 days after they were exposed to plain dendrimer solution or to DAB16-pDNA. Measurement of protein levels by Western blotting was not optimal and the proposals for the improvement regarding e.g. the gels and the extracted protein amounts have been done.

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p53-family, p73, isoforms, regulation, therapeutic targets, Itch ligase, pancreatic cancer

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Tiivistelmä – Referat – Abstract
p53-perheeseen kuuluu kolme transkriptoivaa jäsentä, p53, p73 ja p63. Perheenjäsenillä on yhtälaisia mutta myös yksilöllisiä toimintoja liittyen solujen jakautumiskiertoon, kehittymiseen ja kasvainten syntyyn. p53 ja p73 toimivat pääasiassa tuumorisuppressoreina. Syöpälääkkeen tai säteilyn aiheuttaman DNA-vaurion yhteydessä p53 ja p73 pitoisuudet nousevat ja täten ohjaavat soluja kohti apoptoosia tai solujen jakautumiskierron keskeytymistä. p53:n on havaittu esiintyvän mutatoituneena lähes 50 prosentissa syöpäkasvaimista, estäen täten syöpäsoluja kohtaamasta apoptoosin tai jakautumiskierron keskeytymisen aiheuttamaa solukuolemaa. Toisin kuin p53, p73 ei tyypillisesti esiinny mutatoituneena syöpäsoluissa ja on sen vuoksi potentiaalisempi kohde syövän hoidossa. p73:n kontrollointiin osallistuu useita tekijöitä, joten mahdollisuudet sopivan syöpähoidon kohteen löytämiseen ovat hyvät. Itch-ligaasi kontrolloi negatiivisesti p73:n pitoisuuksia ja on yksi tutkituimmista p73:ta kontrolloivista tekijöistä. p73-tasojen nostaminen rajoittamalla Itch-ligaasin toimintaa on osoittautunut mahdolliseksi geeniterapian lähestymistavaksi. Lisää *in vivo*-tutkimuksia tarvitaan Itch-ligaasin terapeuttisen potentiaalin varmistamiseksi. Syöpäsolujen kohtaloon (solukuolema tai elinkykyisyyden jatkuminen) on havaittu vaikuttavan p73 isoformien (aktivoiva TA-muoto ja negatiivisesti vaikuttava ΔN-muoto) tasapaino ja tasapainon kontrollointi on mahdollisesti myös yksi tulevaisuuden syöpähoidon menetelmistä.

Työn kirjallisessa katsauksessa selvitetään muun muassa p73:n rakennetta, isoformeja, tehtäviä ja mahdollisia siihen liittyviä terapeuttisia kohteita ja kohteisiin kokeltuja lähestymistapoja (*in vivo* ja *in vitro*). Kokeellinen osuus perustuu lähinnä haimasyöpäsoluihin (Mia PaCa-2, PANC1, BxPc-3 ja HPAC) dendrimeeri-nanopartikkeleiden (DAB16-pDNA) avulla tehtävän transfektoinnin onnistumisen arviointiin ja olosuhteiden optimointiin. Myös pelkän dendrimeeriliuoksen ja dendrimeeri-pDNA–kompleksin solutoksisuus testattiin, jotta voitiin arvioida solujen elinkyky transfektoinnin jälkeen. Proteiinimääritykset (p73 ja Itch) tehtiin Western blotting menetelmällä.
Transfektoinnin tulokset haimasyöpäsoluilla osoittautuivat onnistuneiksi (lukuunottamatta BxPc-3-solulinjaa) ja todistivat nanopartikkeleiden toimivuuden mahdollisena geeniterapian välineenä. Solut palautuvat nanopartikkeleiden aiheuttamasta solutoksisuudesta noin kolmen päivän kuluttua nanopartikkeleiden lisäämisestä. Proteiinimääritykset Western blotting-menetelmän avulla osoittautuivat luultua hankalammaksi ja tulokset eivät onnistuneet. Mahdollisia parantamisehdotuksia menetelmän toteuttamiseen on esitetty. Kokeellinen työ on osa suurempaa p73-projektia, jossa päätavoitteena on Itch-geenin poisto (knock-out) syöpäsoluissa siRNAn/shRNA:n avulla ja sen johdosta p73-pitoisuuden nousu. Näin syöpäsolut saataisiin alttiimmiksi syöpälääkkeiden aiheuttamalle solutoksisuudelle.

Avainsanat – Nyckelord – Keywords
p53-perhe, p73, isoformi, kontrollointi, terapeuttiset kohteet, Itch ligaasi, haimasyöpä

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TABLE OF CONTENTS

1 ABBREVIATIONS AND THEIR CLARIFICATIONS

APOPTIN	SMALL PROLINE-RICH PROTEIN
BAX	BCL2-ASSOCIATED X PROTEIN
BD	BASIC DOMAIN
BH3	BCL-2-HOMOLOGY DOMAIN 3
C-ABL	TYROSINE KINASE
CD95	DEATH-RECEPTOR PROTEIN
CHK1/2	CHECK POINT KINASE
CTD	C-TERMINAL DOMAIN
E1A	ADENOVIRAL ONCOGENE
E2F1	TRANSCRIPTION FACTOR
GRAMD4	DEATH-INDUCING PROTEIN
HPV	HUMAN PAPILLOMAVIRUS
HDM2	HUMAN HOMOLOGUE OF MDM2
HELA	CERVICAL CANCER CELL LINE
HIC1	HYPERMETHYLATED IN CANCER 1 PROTEIN
H1299	NON-SMALL CELL LUNG CARCINOMA
IASPP	ONCOPROTEIN (ASPP-INHIBITING)
ID	INHIBITORY DOMAIN
JNK	C-JUN N-TERMINAL KINASE
K562	ERYTHROLEUKEMIC CELL LINE
LSD1	LYSINE-SPECIFIC DEMETHYLASE
MDM2	MURINE DOUBLE MINUTE 2 PROTEIN
MCF-7	BREAST CANCER CELL LINE
MCM7	DNA REPLICATION LICENSING FACTOR
MIRNA	MICRORNA
NEDD4	NEURONAL PRECURSOR CELL-EXPRESSED DEVELOPMENTALLY DOWNREGULATED 4
NETRIN	GUIDANCE FACTOR
N4BP1	NEDD4-BINDING PARTNER-1
PIAS	PROTEIN INHIBITOR OF ACTIVATED STAT
PIN-1	ISOMERASE

PPIG3/DNA	POLYPROPYLENIME DENDRIMER
PRB	RETINOPLASMA PROTEIN
PUMA	P53 UP-REGULATED MODULATOR OF APOPTOSIS
P300/CBP	CO-ACTIVATING PROTEINS
RUNX	RUNT-RELATED TRANSCRIPTION FACTOR
SAM	STERILE ALFA MOTIF
SET9	HISTONE METHYLTRANSFERASE
SHRNA	SMALL-HAIRPIN RNA
SIRNA	SMALL-INTERFERING RNA
SUMO	SMALL UBIQUITIN-RELATED MODIFIER
TA	TRANSACTIVATING
YAP1	YES-ASSOCIATED PROTEIN 1
37AA	SMALL HYBRID PEPTIDE

2 P73

2.1 Main information of p73

2.1.1 Family, structure, cellular location, levels in cancer cells, isoforms

In 1997, Kaghad et al. found a novel monoallelically expressed tumor suppressor gene related to p53 at region 1p36 in several tumor cell lines and named it p73 (Kaghad et al. 1997). p73 shares a significant sequence similarity with the DNA-binding (63% similarity), transactivation (29% similarity) and oligomerization (38% similarity) domains of p53. The homology of p73 with p53 led to assumptions that p73 could play as important a role in cell cycle regulation and growth control as p53. After few additional studies, it became clear that overexpressed p73 activated the transcription of p53-responsive genes and inhibited cell growth by inducing apoptosis (programmed cell death) and cell cycle arrest (Jost, Marin & Kaelin 1997). Now, 14 years later the results from p73 research have indeed proven that p73 possess an important role as a tumor suppressor and may also be one way towards more efficient cancer therapy.

The p53 family consists of transcription factors p53, p63 and p73, which are highly homologous in the previously mentioned domains but differ in their C-terminus (Chi, Ayed & Arrowsmith 1999). p73 and p63 have more similarity in their domain structures when compared with p53. It has been estimated that this tumor suppressor family was generated from a p73/p63-like ancestral gene. The C-terminal domains (CTDs) are the variable parts, possessing tendency towards alternative splicing and post-translational modifications (Sauer et al. 2008). The CTDs affect DNA binding and transcriptional activity of the transcription factor; the different DNA-binding characteristics seem to determine the predominant role of the family members in cellular stress response or in developmental processes. The C-terminus of p53 which has a basic domain (BD) but p63 and p73 have a sterile α motif (SAM) domain and an inhibitory domain (ID) (Chi, Ayed & Arrowsmith 1999). The SAM domain is involved in protein-protein interactions. The structures of p53-family members are presented in Figure 1.

Human p73 transcripts lead to several C-terminal splice variants, from p73α to p73η (De Laurenzi et al. 1998). The p73 gene contains P1 promoter, which produces transactivating TA

isoforms (Killick et al. 2011). An alternative promoter P2 in intron 3 leads to transcription of dominant negative ΔN isoforms, which are able to inhibit the transactivation of the TA-forms.

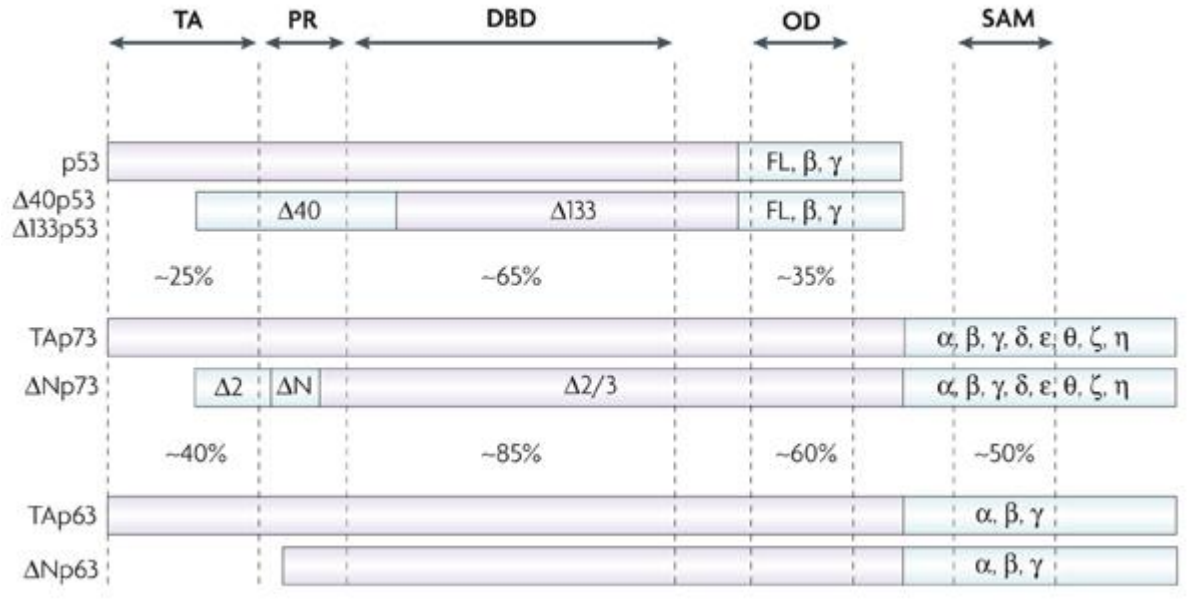


Figure 1. The structures of p53-family members. The basic core is based on a central sequence-specific DNA binding domain (DBD), an N-terminal transactivation domain (TA) and a C-terminal oligomerization domain (OD). p73 and p63 have a SAM-domain. ΔN-forms lack the transactivation domain and show opposing functions compared with TA-forms (Stiewe 2007).

The expression levels of p53-family members are usually upregulated in cancer (Ng et al. 2000); (Choi et al. 2002); (Hong et al. 2007); (Uramoto et al. 2004). Studies have connected upregulation of p73 to the formation of distant metastasis and vascular invasion but the main connection may only be with the dominant-negative isoform of p73 (ΔNp73). This topic will be discussed more detailed later in Chapter 2.

2.1.2 The functions of p73 and response to DNA damage

The p53-family members are connected to several functions in cell cycle regulation, development and tumorigenesis. The family members have similar but also individual roles (Levrero et al. 2000). p53 mainly acts as a tumor suppressor in stress situations and p63 is vital for ectoderm (the embryo layer, where the skin, nervous system and sense organs originate) development. p73 seems to take part in regulating both, the stress responses and developmental processes (e.g. differentiation of neural stem cells) (Agostini et al. 2010). Deficiency of p73 has shown to cause severe neurological and immunological defects in

mice, expecially the lack of dominant-negative isoform ΔNp73, which induces symptoms of neurodegeneration (Wilhelm et al. 2010).

Cells deficient in p73 and p63 are unable to repair DNA damages, e.g. broken DNA double strand (Lin 2009); (Puig et al. 2003). This leads to enhanced tumor formation and progression. p73 has been shown to act also as a T-cell specific tumor suppressor in a mouse model, indicating that the loss of p73 results in an increased incidence of thymic lymphomas when compared to the loss of p53 alone (Nemajerova 2009). This finding reveals a possible role for p73 in the formation of human leukemias and lymphomas.

2.1.3 Apoptosis and cell cycle arrest via p73

2.1.3.1 Apoptosis

Several factors are connected to p73 mediated cell death and the network is complex. The cancer cells undergo DNA damage eg. in response to treatment with DNA damaging agents (e.g. anticancer drugs) or irradiation. This then induces increased levels of the p53 family members. The severity of DNA damage on the cell defines the ultimate result (Bitomsky 2009)**.** When the cell is exposed to a milder DNA damage, it tries to repair itself or goes to a cell cycle arrest. Apoptosis is what happens if the cell is confronted with DNA damage impossible to be repaired.

The observation that most of p73 is localized in the cell's nucleus and is retained there during apoptosis, has led to suggestions that p73 may not have direct effect on the pro-apoptotic factors located outside the nucleus (Melino et al. 2004). It has been proposed an apoptotic pathway controlled p73 would involve p73's direct PUMA (p53 up-regulated modulator of apoptosis) transactivation, which then is capable of directly changing the conformation and mitochondrial relocalization of Bax (Bcl2-associated X protein). This finding proposes a p73 induced PUMA and Bax mediated mitochondrial pathway leading to cell death. The dominant-negative isoforms of p73 have a repressing effect on p73-induced apoptosis. They control the PUMA and Bax pathway negatively and therefore inhibit TAp73- and p53-induced apoptosis.

The JNK (c-Jun N-terminal kinase) is an important mediator of the stress response in DNA damaged cells (Jones, Dickman & Whitmarsh 2007). There has been found a link between

p73 and JNK during exposure to chemotherapeutic agents (e.g. cisplatin). Studies by Jones et al. show, that JNK creates a complex with p73 and phosphorylates p73 at numerous amino acid residues (e.g. serine, threonine), leading to stabilization of p73, enhanced p73-transcriptional activity, and apoptosis. More specifically, JNK causes stabilization and apoptotic effects through TAp73 isoform while at the same time promoting the degradation of antiapoptotic ΔNp73 isoform (Dulloo et al. 2010). The tyrosine kinase c-Abl has also been shown to phosphorylate p73 when cells are exposed to DNA damage i.e. caused by gamma-irradiation or DNA damaging agent (e.g. cisplatin) *in vitro* and *in vivo* (Agami et al. 1999). These two kinases, JNK and c-Abl regulate p73 apoptotic response during DNA damage and therefore are important modulators of a cytotoxic response.

2.1.3.2 Cell cycle arrest

p73 induces cells to apoptoses and also causes cell cycle arrest. First studies, carried out in 1998 by Zhu et al. in H1299 (non-small cell lung carcinoma) cells showed that p73-dependent cell cycle arrest occurs in both the G as well as G2-M phase similar to that induced by p53 (Zhu et al. 1998) (Cell cycle phases are shown in Figure 2).

Only few studies have analysed the regulation of p53-family proteins during cell cycle progression. The amount of these proteins is different depending on the cell cycle phase (Lefkimmiatis et al. 2009). When MCF-7 (breast cancer) cells were examined, the concentration of TAp73α isoform was higher in S phase cells, whereas ΔNp73α was at highest level in G1-S phase transition and in the beginning of S phase. This suggests that the upregulated levels of p73 seen in cancers may support abnormal cellular proliferation (via growth progression genes involved in the cell cycle phases) and play a part in cancer initiation or growth.

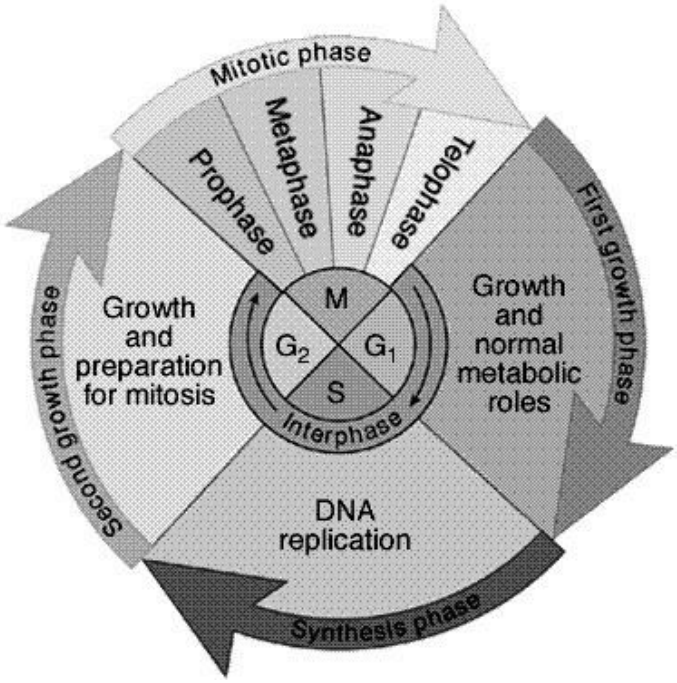


Figure 2. Cell cycle with different phases. p73-mediated cell cycle arrest takes place during G1 phase and from G2 to M phase. G1 phase is a growth stage, allowing cell to produce RNAs, proteins and other cellular molecules. In G2 phase cell duplicates chromosomal proteins and DNA. In M phase the cell goes through mitosis at four stages; prophase, metaphase, anaphase and telophase (St. Rosemary Educational School 2010)**.**

2.2 p73 mutations in cancer cells and the effect of p53 mutations to p73 function

Usually the loss of heterozygosity in tumors is found in the 1p chromosome region, where also various tumor suppressor genes are located (Han et al. 1999). Unlike p53, which is mutated almost in half of the human cancers, p73 appears to be mutated in human cancers when various types of tumors have been investigated, including breast, colorectal, gastric, lung, pancreatic cancer and neuroblastomas (Hollstein et al. 1994); (Yoshikawa et al. 1999); (Takahashi et al. 1998); (Mai et al. 1998); (Nomoto et al. 1998). When originally discovered no mutations of p73 were found in 15 cancer cell lines (Kaghad et al. 1997). p53 mutations and p73 mutations together in same cell line have only been found in 2 out of 17 lung cancer cell lines. (Yoshikawa et al. 1999).

Even though the overall amount of mutations is small, a few have been identified. A mutation of p73α (G264W) was shown to have a suppressing effect on the transactivating wild-type p73α, when three naturally occurring p73 mutants were examined from lung cancer cell lines (Huqun et al. 2003). The p73 mutation P425L was seen to reduce the ability to activate p73

and p53 regulators (e.g. Bax, MDM2) (Naka et al. 2001). The biological significance of these p73 mutations clearly needs more studies. The fact that mutations in both p53 and p73 genes rarely occur simultaneously emphasises the family members's individual roles in cancers and the narrow mutation profile of p73 is very positive when considering for anticancer therapy.

In cells that have mutated p53 the function of tumor suppressor p53-family members is particularly important. In the presence of mutant p53, at least p73 has been shown to preserve the cell growth suppression through induction apoptosis and cell cycle arrest, a p21-mediated downstream pathway has also been proposed (Willis et al. 2003). However, mutant p53 proteins are also capable of binding and down regulating several p73 and p63 isoforms (both TA- and ΔN-forms, from α to Δ) resulting to inhibition of p73 and p63 functions (Gaiddon et al. 2001). The inhibition level depends on the binding efficiency. Specific DNA binding and the oligomerization domains in p73 are required for the interaction of p73 with mutant p53. In the case of mutant p53, only the core domain is needed for the interaction (Strano et al. 2000).

3 P73 AND ITS REGULATION, INTERACTIONS AND ROLE IN CHEMOSENSITIVITY

3.1 p73 regulators

The network surrounding p73 is extensive, consisting of at least 53 proteins and 176 interactions with different functions from p73 upregulation to its transcriptional activation (Tozluoğlu et al. 2008). The categories are shown in Table 1. This chapter will clarify the factors controlling the function of p73.

Table 1. Proteins connected with p73 grouped into functional categories, consisting of upregulation, activation, suppression, degradation and transcriptional activation. One protein may be in many categories, if it shows different functions. p73 isoform ΔN and factor pRB are put separately, because their role is uncertain in several paths (Tozluoğlu et al. 2008)

Functional categories				
Upregulation	Activation	Suppression	Degradation	Transcriptional activation
E2F1	Pin-1	RACK1	Cyclin G	hTERT
Chk2	c-Jun	MDM2	Ubc-9	Apaf-1
TGFβ	ASPP1/2	MDMX	Cullin	PUMA
ZEB	p38	CTF2	Itch	Bax
Tax	PML	WT1	NQO1	Scotin
E1A	MM1	SIRT1	UFD2a	p21
	c-Abl	E4orf6	Roc1	GADD45
	YAP	Akt		14-3-3σ
	p300/CBP	PIAS-1		IGF-BP3
	Chk1	Daxx		GADD 153/CHOP
		Amphiphysin		
	$p19^{ras}$	IIb-1		Noxa
pRB	PKCδCF	Wwox		Killer/DR5
p73	ATM	Cyclin		PIG3

		A/B:CDK1/2		
ΔNp73	ATR	PKA-Cβ		CD95
	PMS2	Pirh2		p53AIP1
	NEDL2			p53R2
	HIPK2			
	PML-NB			

3.2 Upregulation by E2F1, EA1, Chk1/Chk2

E2F1 is a nuclear transcription factor which has an oncogenic as well as pro-apoptotic properties. It is involved in the control of the cell-cycle, specifically the transition from G1 phase to S phase (Tozluoğlu et al. 2008). Phosphorylation of E2F1 is the key mechanism causing its activation. E2F1 acts as a direct transcriptional regulator for p73 and intracellular levels of E2F1 and p73 are important factors determining cell fate (Ozaki 2009). The transcriptional activity of p73α is due to E2F1's amino acid residues 1-117. Amino acid residues 118 to 285 of E2F1 have an essential role in the regulation of p73α expression levels.

E2F1 controls p73 in the presence and absence of DNA damage (Urist et al. 2004). The E2F1 induced expression of p73 leads to activation of p53-responsive target genes and to cell cycle arrest or apoptosis (Stiewe, Putzer 2000); (Tozluoğlu et al. 2008). Deregulated expression of E2F1 leads to proteolytic degradation of p73 in a proteasome-independent manner: The deregulation of E2F1 is a common genetic alteration in human tumours (Bell, Ryan 2004). The activity of E2F1 can be inhibited by it forming a complex with pRB (a retinoplasma protein, tumor suppressor) (Tozluoğlu et al. 2008).

E1A is an adenoviral oncogene that when added to the cancer cells (studied e.g. in head and neck cancer cell lines), it significantly increases the expression of transcriptionally activating p73 isoform TAp73 but does not have any effect on the dominant negative suppressive isoform ΔNp73 (Flinterman 2005). In the absence of functional p53, E1A and Apoptin (viral proteins added to cancer cells) have the ability to activate apoptotic pathways involving p73 and p53 targets, and induce efficient tumor cell death (Klanrit et al. 2008).

The protein kinases checkpoint kinase Chk1 and Chk2 are central in the induction of cell cycle arrest, DNA repair and apoptosis (Tozluoğlu et al. 2008). p53 and p73 have a similar pathway and affect Chk1 and Chk2 but by different mechanisms. In p73 control, Chk1 activates p73 and Chk2 is more evolved in p73 upregulation. These protein kinases control p73 levels after DNA damage in several human tumor cell lines (Urist et al. 2004). Ckh1 and Chk2 also regulate the stability and activity of E2F1 after genotoxic stress and through this pathway they have an effect on TAp73 transcription.

3.3 Activation by c-Abl, JNK and ASPP1/ASPP2

Activation of the tyrosine kinase c-Abl results either in cell cycle arrest in phase G1 or in apoptotic cell death (Agami et al. 1999). When DNA damage is recognized c-Abl is activated by phosphorylation. After phosphorylation it binds to PXXP- motif of p73 and further phosphorylates p73 at the tyrosine and threonine residues. This results in a signaling cascade involving e.g. p38 and Pin-1 isomerase, (Tozluoğlu et al. 2008), which leads to the induction of p73-mediated apoptosis (Yuan et al. 1999).

JNK (c-Jun N-terminal kinase) acts mainly as a positive regulator of p73 by inhibiting p73 degradation and stimulating p73 transcriptional activity (Tozluoğlu et al. 2008). Paradoxally, JNK also binds to MDM2 and induces its transcription, which suppresses p73 activity. The role of JNK in p73-network seems to be complex, because it has been seen to promote ΔNp73 degradation through a ubiquitin-independent but proteasome-dependent mechanism, activated in situations involving genotoxic stress (Dulloo et al. 2010). The apoptosis during treatment with DNA-damaging agents has been shown to involve the complex formation between JNK and p73, leading to phosphorylation of p73 at numerous amino acid residues (e.g. serine, threonine) (Jones, Dickman & Whitmarsh 2007). This event leads to p73 protein stabilization, enhanced p300-mediated acetylation of p73 and increased p73-mediated transcriptional activity and apoptosis.

ASPP1 and ASPP2 are proteins that can induce apoptosis by binding to p53 family members and selectively induce the expression of endogenous p53 target genes (for example PIG3, PUMA)(Bergamaschi et al. 2004). They also stimulate the transactivation function of p73 and p63 on the promoters (e.g. Bax, PUMA).

3.4 Degradation and suppression by Itch, YAP1, PIAS, MDM2 and iASPP

Itch is an ubiquitin-protein isopeptide (E3) ligase and it is a key controller of p73 protein levels (Rossi et al. 2005). Itch controls the levels of p73 by selectively binding to p73 by PY-motif and by ubiquitinating it. This leads to a proteosome-dependent degradation of p73. During DNA damage Itch levels are downregulated, allowing p73 levels to increase. The role of of Itch in p73 degradation is similar to that of MDM2 in p53 pathway (Tozluoğlu et al. 2008).

Yes-associated protein 1 (Yap1) has been shown to bind to p73 by the same PY-motif and increase p73 transactivation of apoptotic genes (Levy 2007). Under normal conditions, YAP1 makes a complex with co-activator Runx and the complex binds to Itch promoter and supports Itch transcription leading to degradation of p73 (Levy, Reuven & Shaul 2008). But in situations involving DNA damage, a tyrosine kinase c-Abl phosphorylates Yap1 and the Yap1-Runx complex does not develop and the p73 degradation effect by Itch is not supported. Leading to p73 accumulation this Yes-associated protein 1 has thus an important role regulating p73 levels during DNA damage.

PIAS (protein inhibitor of activated STAT) proteins interact and modulate the activities of various transcription factors and function as SUMO (small ubiquitin-related modifier) ligases by sumoylating targets (Kotaja et al. 2002). Sumoylation is a similar process as ubiquitination but it does not lead to protein degradation, instead via PIAS proteins it affects target's transcriptional activity on promoters. It has been shown that a RING finger (functional domain) of PIAS-1 protein binds to p73 and sumoylates it (Munarriz et al. 2004). This phenomenon decreases the p73 transcriptional activity on promoters, for example Bax, and is an important factor in the cell cycle regulation. The reduced expression of PIAS1 protein has been associated for example with colon cancer development (Coppola et al. 2009).

MDM2 is an E3 ubiquitin ligase, which is an important factor in p53 network but also affects p73. It is transcriptionally activated by p73 and, in turn, negatively regulates the function of p73 (Zeng et al. 1999). The inactivation of p73 by MDM2 does not occur via a degradation pathway (Tozluoğlu et al. 2008), instead MDM2 has the ability to disrupt the interaction of p73 with p300/CBP proteins by competitive binding (Zeng et al. 1999). Only recently it has been found that MDM2 indirectly induces the degradation of p73 through interaction with

Itch ligase (Kubo et al. 2010). Interaction of MDM2-Itch was seen only in HeLa cells but not in H1299 cells, indicating that this observation clearly needs more evidence. Also MDM family member MDMX binds to p73 and affects its levels (Ongkeko et al. 1999).

There is an inhibitory member of ASPP protein family connected with p53 family members, an oncoprotein called iASPP. It functions as a negative regulator of p73, p53 and p63 (Robinson et al. 2008). iASPP cooperates with p73 regulators, for example with E1A (Bergamaschi et al. 2003).

3.5 Transcriptional regulation by PUMA, Bax, Noxa, CD95 and GRAMD4

p73 transcriptionally activates a number of genes which are involved in cell cycle control and apoptosis. Some of the target genes are in common with other p53 family members and this chapter will briefly introduce a few of the main target genes which have been connected with p73 without going into the similarity of target genes with other p53 family members.

PUMA belongs to the BH3-only Bcl-2 family of apoptotic regulators (Nakano, Vousden 2001). In normal conditions the level of PUMA is kept low but up-regulated, for example by p53, p73 and E2F1, it causes programmed cell death. PUMA controls apoptosis by regulating the release of pro-apoptotic factors from cell's mitochondria. It also causes conformational change of Bax (Bcl-2 family member) and activates its mitochondrial translocation (Bcl-2 family member)(Tozluoğlu et al. 2008). Cytochrome c release is follows after the mitochondrial translocation of Bax, leading to activation of the caspase cascade and eventually to apoptosis (Melino et al. 2004); (Tozluoğlu et al. 2008). It seems that the p73-mediated induction of PUMA happens through p73β (Sun 2009). p73 induces also the expression of apoptotic Noxa gene after genotoxin treatment, also leading to apoptosis (Martin et al. 2009).

CD95 is a gene that encodes for a cell's death receptor (Schuster 2010). It is regulated by p73 isoforms TAp73β and ΔNp73 and the effect of each isoform leads to an opposite result (Muller 2005). TAp73β directly activates the CD95 via the p53-binding site and also induces the expression of proapoptotic Bcl-2 family members in mitochondria. Instead, isoform

ΔNp73 inhibits the CD95 transactivation and apoptosis and by doing so, inactivates the tumor suppressor function of TAp73β and also p53.

An important and a novel pathway of p73-induced apoptosis was introduced by John et al in their studies in 2010 (John et al. 2010). They showed that p73-induced apoptosis after the DNA-damage caused by chemotherapeutic drug is partly mediated by GRAMD4 protein (alias Death-Inducing-Protein) expression and translocation to the mitochondria. GRAMD4 physically interacts with Bcl-2, promotes Bax mitochondrial relocalization and oligomerization, which proceeds to other events leading to apoptotic cell death.

3.6 The interactions between the p53 family members p53, p73 and p63 in cancer

The p53 family members have compensatory mechanisms with each other, ensuring that apoptotic functions are maintained in cells that lack some family member, e.g. in p53-deficient cells. The ΔN isoforms of family members act by suppressing the functions of TA (transactivating) isoforms. A concominant loss of two family members, p73 and p63 has been seen to result in failure of apoptosis in fibroblasts, even though the cells had functional p53 (Flores et al. 2002). The result indicates the crucial role of other family members in p53-dependent apoptosis. In T-cells the dependence of p63 and p73 for p53-mediated apoptosis was not as clear, indicating a cell type specificity for p53-family interactions (Senoo et al. 2004).

3.6.1 Compensatory roles

3.6.1.1 p53 and p73

p53 and p73 have also shown to have an antagonistic activities with each other in acute myeloid leukemia cells via protein kinases Chk1 and Chk2 (Chakraborty 2010). In p53-impaired cancer cells DNA damage causes accumulation of p73 via Chk1-mediated pathway, also by downregulation of dominant regulative isoform of p73 (ΔNp73). By contrast, when p53 function is present, Chk2 induces upregulation of p53 which overshadows p73 activity. This means that there is a compensating mechanism for apoptosis between p53 family members p53 and p73.

3.6.1.2 TAp63/ ΔNp63 and p53

Compensatory mechanisms are also involved between p63 and p53, with respect to cell cycle regulation and DNA damage repair in cancer cells (Yao, Chen 2010). In p53-deficient cancer cells expecially the transactivating p63 isoform TAp63 expression upregulates under genotoxic stress caused by chemotherapeutic drug. The p53-expressing cells have reduced TAp63 promoter activity.

3.6.2 Suppressing roles

3.6.2.1 ΔNp63α and p73

The p63 isoform ΔNp63α has an important role in suppressing p73-dependent apoptosis, especially in cancer cells that overexpress this isoform (Rocco et al. 2006). ΔNp63α inhibits p73-dependent transcription by direct promoter binding and also by physical interaction with p73. The expression level of ΔNp63α has been proven to be an essential survival factor in head and neck squamos cell carcinoma (HNSCC) (Rocco et al. 2006).

3.6.2.2 ΔNp73 and p53/Tap73

The tumor suppressor action of p53 and TAp73 can be inactivated by ΔNp73 to block their apoptotic function (Muller 2005); (Grob et al. 2001). In the case of TAp73 specifically the form TAp73β is inhibited by ΔNp73. Both effects originate from the mitochondria and inhibition of CD95 gene transactivation has been proven to be one of the mechanisms involved. Naturally, the ΔNp73 is upregulated by the TAp73 and p53. This proves that a feedback loop that tightly regulates the functions of TAp73 and p53 exists. ΔNp73 may also have a role as a prognostic factor in cancer because upregulated ΔNp73 may be a sign of reduced survival possility (Dominguez et al. 2006).

3.7 The interacting p53-family contributes to chemosensitivity and – resistance

Several studies have shown that p73 levels are upregulated in cancer cells in response to DNA damage caused by chemotherapeutic agents (Muller et al. 2006). Recently Seitz et al. studied the important role of p53 family members in resistance of hepatocellular cancer cells

to chemotherapeutic drugs (Seitz et al. 2010). The study revealed that all endogenous p53 family members (p53, p63 and p73) are upregulated in response to DNA damaging agents and by blocking the p53 family function cancer cells become chemoresistant. Chemotherapeutic treatment induced the expression of proapoptotic (e.g. Bax) but also antiapoptotic (e.g. MDM2) factors in p53-family mediated downstream pathways. This indicates that the final result, sensitivity or resistance to chemotherapy depends on the delicate balance between previously mentioned factors.

More precisely, p73 isoform TAp73 has been connected to chemosensitivity in hepatocellular carcinoma (Muller 2005). It is upregulated in cancer cells after treatment with anticancer agents. In contrast, ΔNp73 is causing the resistance to chemotherapy. The cellular response to the chemotherapeutic treatment is unquestionably more dependent on the entire activity of p53-family, rather than one member alone (Vilgelm et al. 2010b).

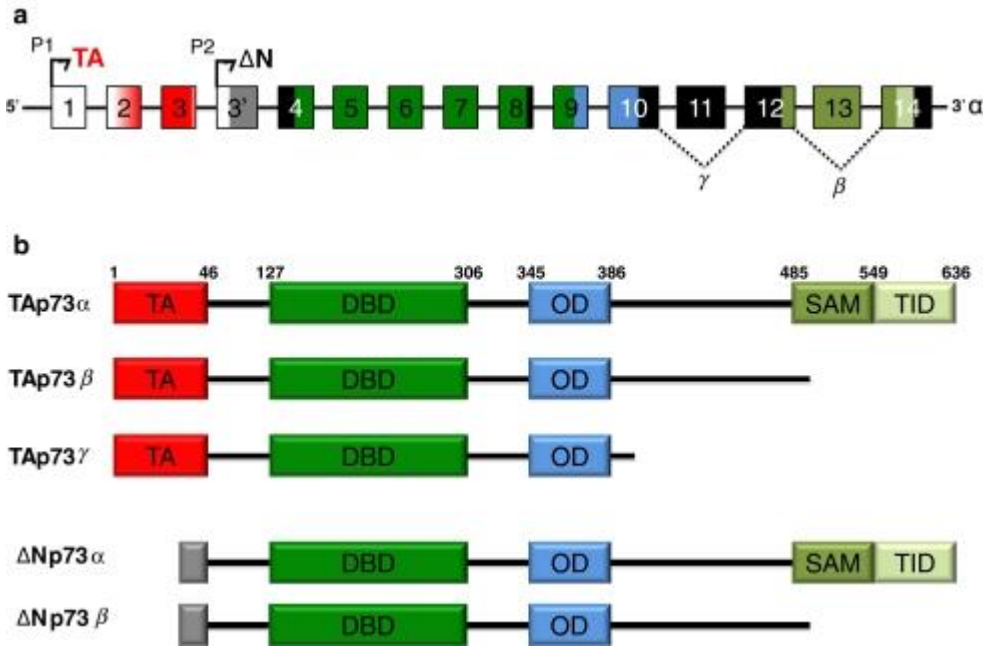
4 ROLES OF P73 ISOFORMS IN CANCER

4.1 TAp73 and ΔNp73

4.1.1 Structures

p73 has several isoforms with differences in function (Koeppel et al. 2011). The main p73 isoforms are transactivating TA-forms and aminoterminally truncated ΔN-forms. The p73 gene contains the P1 promoter, which produces TA isoforms (Killick et al. 2011). An alternative promoter P2 in intron 3 leads to transcription of the ΔN isoforms which are able to inhibit the transactivation of the TA-forms (Grob et al. 2001); (Zaika et al. 2002). TA- and ΔN-forms have opposite roles showing pro-and antiapoptotic functions and the balance between these isoforms is the determining the cell fate.

The transactivating TA-isoform have different C-terminal splice forms (subtypes) e.g. α and β (Koeppel et al. 2011). These subtypes have different DNA-binding capacity and these functions are diverse depending on the tumor cell background (Holcakova 2008). The aminoterminally truncated ΔN-forms, also called ΔTAp73 isoforms, exist as ΔNp73, ΔN'p73, Ex2p73 and Ex2/3p73 (Concin et al. 2004). ΔNp73 is called a dominant-negative isoform because it lacks the transactivation domain of full-length TAp73 (Zaika et al. 2002).



Picture 3. The structure of p73 gene. **a** Genomic organisation of p73 and the splicing variants that behind the isoforms of p73. The P1 promoter generates the TA isoforms, while the P2 promoter produces the ΔN isoforms. **b** Schematic representation of the domains encoded by the different isoforms of p73. On top, there are indicated the aminoacids included in each domain. *TA* transactivation domain, *DBD* DNA-binding domain, *OD* oligomerization domain, *SAM* SAM domain, *TID* transactivation inhibitory domain (Killick et al. 2011).

4.1.2 Location, expression levels and specific regulators

While TAp73 and ΔNp73 are mainly located in the cell nuclei, some small amounts are also found in the cytoplasm (Nekulova et al. 2010). The levels of p73 isoforms are usually higher in tumors than in normal tissue (Faridoni-Laurens et al. 2008). The expression levels of the NH_2- truncated isoforms of p73 are higher in cancer patients (e.g. in neuroblastoma, ovarian, lung, colon and breast) than in healthy individuals (Concin et al. 2004); (Dominguez et al. 2006) ; (Uramoto et al. 2004). However, TAp73 expression levels show differences between cancer types. In ovarian cancer approximately 30 per cent of the patients have also higher TAp73 levels, but for example in neck and head squamous carcinoma TAp73 is only weakly expressed (Faridoni-Laurens et al. 2008).

The balance between the levels of p73 isoforms, i.e. proapoptotic TAp73 and anti-apoptotic ΔNp73, is a key determinant of a cell fate. Studies have shown the regulatory differences of p73 isoforms, even though the number of studies seems to still be minimal. The expression of endogenous TAp73 is induced at least by E2F and by viral proteins E1A and Apoptin

(Waltermann 2003); (Flinterman 2005) ; (Klanrit et al. 2008). The induction of TAp73 via E1A and Apoptin activates pro-apoptotic target PUMA, independently of p53.

One specific TAp73-mediated mechanism towards apoptosis is through CD95 gene (Muller 2005). TAp73 transactivates the CD95 gene via the p53-binding site in the first intron and induces the expression of proapoptotic Bcl-2 family members (e.g. PUMA), leading to apoptosis. The regulation of TAp73 proteins has been linked to protein family of Netrins and their receptors (e.g. receptor DCC, deleted in colorectal cancer) (Roperch 2008). Netrins have a wide role in axon guidance, cancer cell survival, tumor angiogenesis and metastasis. Netrin-1 selectively raises TAp73α protein levels by preventing ubiquitination and degradation but does not have any effect on ΔNp73α protein or to the transcripts encoding TAp73α or ΔNp73α. The results were seen in human cervical cancer cells.

Induction of the levels of ΔNp73 is partly due to deregulation of the E2F1-responsive P1-promoter. In contrast to the TAp73 induction, E1A does not have an effect on the levels of ΔNp73 (Concin et al. 2004) ; (Flinterman 2005). The regulation of ΔNp73 has been connected also to a region within the ΔNp73 gene promoter, containing a binding position for HIC1 (Hypermethylated In Cancer 1 protein) (Vilgelm et al. 2010a). HIC1 has been shown to negatively regulate ΔNp73 transcription in mucosal epithelial cells, taken from gastric and esophageal tumors. The negative regulation resulted in reduction of ΔNp73 levels.

ΔNp73 is capable of acting as an inhibitor of wild-type p53 and TAp73, by inhibiting transactivation function and apoptosis mediated by wild-type p53 and TAp73. It also shuts off its own expression so that it can finely regulate the whole p73 system (Grob et al. 2001). One mechanism which clarifies the inhibitory function of ΔNp73 is that it competes for the DNA binding site with p53 and directly associates with TAp73 (Ishimoto et al. 2002). The drug resistance in wild-type p53 tumor cells is also caused by ΔNp73 (Zaika et al. 2002).

4.2 p73 isoforms connected to cancer prognosis and survival

p73 is associated with tumor subtypes, clinical outcomes and responses to therapy and the levels of its isoforms may serve as markers predicting the future outcome for the cancer patient e.g. (Weber et al. 2002). It is clear that the ratio of p73 isoforms, TAp73 and ΔNp73 with their opposite functions determines the final cell fate (Tomasini 2008). The upregulation

of ΔTAp73 variants has been linked to advanced pathologic stage, lymph node metastasis and vascular invasion in colon and breast cancer (Concin et al. 2004); (Dominguez et al. 2006); (Uramoto et al. 2004). In other cancers poor prognosis, metastasis and advanced tumor stage have been mainly correlated with higher expression of ΔTAp73 variants (Dominguez et al. 2006).

In neuroblastome the ΔNp73 expression level correlates with poor outcome, independent of age, primary tumor site and stage (Casciano et al. 2002), the lower levels of ΔTAp73 variants (ΔNp73 and ΔN´p73) predict better overall survival. Mice generated specifically deficient for ΔNp73 has shown a decrease in size of a tumor without having an effect on apoptosis (Wilhelm et al. 2010). The loss of ΔNp73 prevents the transformed cells from iniating a new tumor.

The combination of p53 mutational status with p73 isoforms expression have clinical significance for drug resistance (Concin et al. 2005). Patients having p53 mutation together with high levels of ΔTAp73 variants (ΔNp73 and ΔN´p73) were more likely to have chemotherapeutic failure (platinum-based therapy) and had worse overall survival. Interestingly patients with p53 mutation that efficiently inhibited TAp73 function had also significantly shorter overall survival than patients with unknown p53 mutation. The expression levels of p73 isoforms also play a role in response to radiation therapy (Liu et al. 2006). Overexpression of ΔNp73 found in cervical cancer caused resistance to irradiation, whereas TAp73α upregulation was seen in radiation-sensitive cancer tissue. The recurrence of the disease was much more evident when ΔNp73 was upregulated whereas TAp73α predicted better survival.

The expression levels of p73 and its isoforms can correlate with prognosis but significant survival differences have also been observed between the p73 expression alone and p73 coexpressed with p63, at least in bile duct carcinoma (Hong et al. 2007). In tumors that expressed both p73 and p63, the median survival was 13 months while with tumors only expressing p73 the median survival was 35 months. The mechanism behind p73 and p63 causing the poorer survival may be linked to interaction of these isoforms. The interaction of ΔNp63 and TAp73 can be one factor leading to the suppression of apoptosis and potential oncogenic effects (Rocco et al. 2006). In recent reports the balance between the p73 and p63 isoforms in cancer prognosis have also been studied, indicating an more important role for an

altered ratio of p73 and p63 isoforms than for the overexpressed levels of individual isoforms (Iacono et al. 2011).

4.3 How to distinguish between isoforms

Proper detection of and discrimination between the tumor suppressor family members of p53 and their different isoforms in human tissues, especially in tumors is becoming more important in cancer research and in clinical care (Rosenbluth 2009). Commercial antibodies exist to distinguishing p53 family members and e.g. isoforms TAp73 and ΔNp73. The methods used include of western blot analysis, immunochemistry and immunofluorescence. Rosenbluth et al. generated polyclonal mouse and rabbit antibodies which are highly specific for ΔNp73 protein isoforms without p63 cross-reactivity. The group also found p73 antibody Ab4 that is highly specific for α and β C-terminal isoforms of p73 (Tap73α/β) without confusing it with other family members. The levels and intracellular distribution of p73 isoforms TAp73 and ΔNp73 can successfully be detected by immunocytochemical staining (Nekulova et al. 2010).

5 THERAPEUTIC TARGETS IN THE P73 PATHWAY AND THEIR POTENTIAL CLINICAL VALUE

5.1 Potential therapeutic targets

5.1.1 Itch ligase

The protein ligase Itch belongs to the NEDD4-like family of ubiquitin E3 ligases (Rossi et al. 2005). It is connected to several functions in animals and humans and to multiple signaling pathways and involvement in pathological conditions (Melino et al. 2008). Itch deficiency in humans has been shown to cause e.g. multisystem autoimmune disease and developmental abnormalities (Lohr et al. 2010). Itch deficient mice (Itchy mice) possess severe immune and inflammatory defects (lung and stomach inflammation, hyperplasia (overgrowth) of lymphoid and hematopoietic cells) together with persistent scratching of the skin (Perry et al. 1998).

Itch targets transcriptional regulators and, by doing so, affects cell growth, differentiation and apoptotic processes (Melino et al. 2008). The ligase selectively binds to the protein's PY-motif, this leads to ubiquitination of p73 and p63 but not p53, because p53 does not possess the specific PY-motif for binding (Rossi 2006); (Rossi et al. 2005). Instead the ubiquitination and degradation of p53 is regulated by its transcriptional target MDM2 (Wang, Wang & Jiang 2011). Ubiquitination of the proteins then leads to their rapid proteasome-dependent degradation and is an important mechanism for control of the proteins' steady-state levels. Itch affects both p73 isoforms, TAp73 and ΔNp73 (Rossi et al. 2005). C-terminal isoforms of p73, α and β are also under its regulation, while δ and γ lack the PY-domain and are not influenced by Itch.

Under normal conditions, Itch maintains the levels of p73 in balance in cells. After the DNA damage (e.g. cancer treatment or γ-irradiation) the levels of Itch are down regulated, allowing the tumor suppressor levels to stabilize. In cancer cells the Itch down-regulation by DNA-damaging agents (e.g. doxorubicin) has been shown to be time- and dose-dependent. At the same time as the Itch levels go down, the levels of p73 isoform TAp73 levels rise, while ΔNp73 levels reduce, making the Itch regulation an interesting therapeutic target.

In the near future Itch may be used as a therapeutic target in cancer therapy. Down regulating the Itch levels could make cancer cells more sensitive to anticancer agents via upregulated p73 apoptotic function and improve the efficacy of the therapy (Hansen et al. 2007). Especially cells which lack functional p53 are more sensitive to Itch down regulation, indicating that Itch depletion could work in cancers which lack p53 or where it is mutated. p73 levels have been also shown to decline more slowly after siRNA-mediated reduction in Itch levels (Rossi et al. 2005).

5.1.2 ASPP1, ASPP2 and iASPP proteins

ASPP1 and ASPP2 belong to ASPP-family of proteins, which have proapoptotic effects (Samuels-Lev et al. 2001). ASPP1 is a C-terminal half of ASPP2 and a homologous to a protein called 53BP2. ASPP1 and ASPP2 bind to p53 family members *in vitro* and *in vivo* and stimulate their apoptotic function (Bergamaschi et al. 2004). The stimulating effect comes through promoters of proapoptotic genes, e.g. Bax and PUMA (Samuels-Lev et al. 2001). ASPP proteins don't have any effect on cell cycle arrest. The expression of ASPP proteins is often down regulated in cancer, lower levels found e.g. from colorectal cancer, breast cancer and osteosarcoma (Bergamaschi et al. 2004). In breast carcinoma ASPP proteins were down regulated in 60 % of the tumors and only in carcinomas expressing wild-type p53 but not in those with mutant p53.

Inhibitory member of the ASPP family have also been identified, an oncoprotein called iASPP (Bergamaschi et al. 2003). iASPP competes with both ASPP proteins of the binding with p53-family members and this way inhibits apoptosis (Robinson et al. 2008). iASPP cooperates with p73 regulators, for example with E1A (Bergamaschi et al. 2003). It potentiates resistance for cytotoxic drugs and for UV-radiation in tumors (Bergamaschi et al. 2003). In some tumors iASSP has been found to be overexpressed, mainly in tumors expressing wild-type p53. Inhibiting the oncogenic function of iASSP could act as a way to improve cancer treatment at least for tumors expressing wild-type p53.

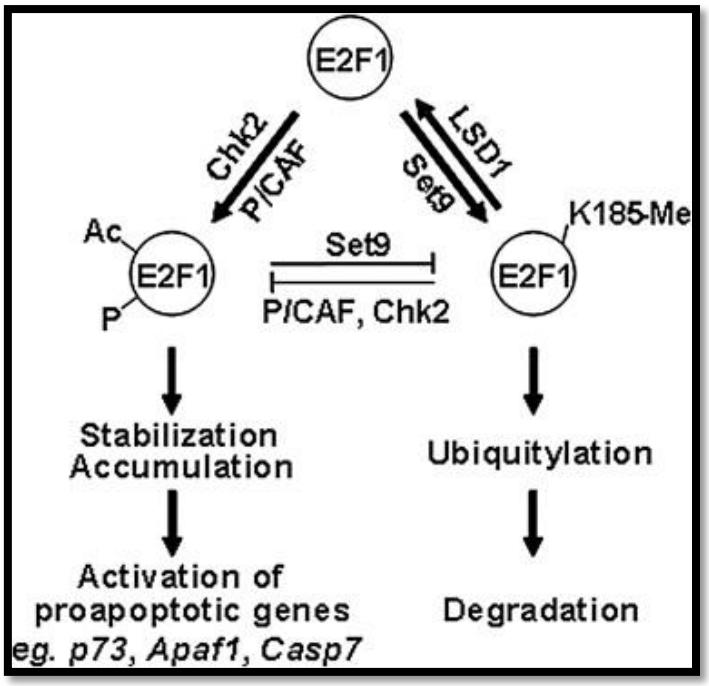
In 2007 Bell et al. described a minimal p53-derived peptide (composed of 37 amino acids) that induces apoptosis by binding to iASSP protein, regardless of the tumor cell's p53 status (Bell et al. 2007). By binding to iASPP in p53-null cells the peptide prevents the interaction between iASPP and p73, leading to activation of the endogenous p73 target genes and finally

to p73-mediated cell death. The effect was seen *in vitro* and *in vivo* in several tumor cell types but not in normal cells. This 37AA peptide offers potential therapeutic tool to induce the p73 expression and enhance the tumor suppressing effect.

5.1.3 Transcription factor E2F1

E2F1 is an important factor in DNA damage-induced apoptosis and its post-translational modification and the control of its expression levels may affect p73-mediated pathways to enhance cell death. Regulating the E2F1/p73 pathway is one method to improve the apoptotic effects in cancer cells (Rodicker et al. 2001); (Helgason, O'Prey & Ryan 2010). In studies of Rödicker et al. the upregulation of E2F1 by using intratumoral injection of an adenovirus vector (based on the plasmid) expressing E2F1 led to the induction of p73 and to a more powerful apoptotic outcome when combined with administration of chemotherapeutic drug (Rödicker F et al. 2001). In pancreatic cancer the response to therapy was seen as a reduction in tumor volume. Also in melanoma, cancer cells exposed to topoisomerase II drugs with adenovirus mediated E2F1 increased apoptosis *in vitro* and *in vivo* (Dong et al. 2002).

Methylation and demethylation of E2F1 are posttranslational events, that regulate the intracellular protein levels of E2F1 in respect to DNA damage (Xie et al. 2011); (Kontaki, Talianidis 2010). Methylation prevents acetylation and phosphorylation of the E2F1 protein, processes which are important e.g. for the activation of protapoptotic p73. During DNA damage the methylation of E2F1 is strongly reduced and the stabilizing demethylated effects take over, leading to apoptotic responses. Lysine-methyltransferases Set9 and LSD1 have opposing functions and regulate whether E2F1 goes through degradation or stabilized accumulation. The Set9- and LSD1- mediated pathway is demonstrated in Figure 4. Cancer therapy combining DNA-damaging agents and drugs which control Set9 and LSD1 could result to a more effective cancer cell death.



Picture 4. In p53-deficients cells, lysine-methyltransferases Set9 and LSD1 regulate whether E2F1 goes through its degradation or activation of proapoptotic genes e.g. p73 during DNA damage. Set9 and LSD1 have opposing functions. Set9 methylates E2F1 which leads to inhibition of E2F1 accumulation and apoptotic responses. LSD1 removes Set9 and starts activation of apoptotic genes (Kontaki, Talianidis 2010).

5.2 p73 isoforms as therapeutic targets

As discussed previously, the relative ratio of each p73 isoform (TAp73 and ΔNp73) is a critical factor in determining the cell's fate, as they have opposing effects on cell death. A mechanism for regulating the levels of these isoforms can enhance the cancer cells to become more sensitive to chemotherapeutic treatment and inhibit tumor growth (Sayan 2010) ; (Emmrich et al. 2009). Results to date show, that events and factors leading to proteasomal degradation of p73 in most cases do not distinguish between the two isoforms.

Savan et al. found a ring finger ubiquitin ligase named as PIR2, which is able to produce an isoform-specific outcome (Sayan 2010). During DNA damage, PIR2 relieves the inhibitory effect of ΔNp73 on TAp73 by favoring degradation of ΔNp73 and in this way increases the ratio of TA/ΔN to a more apoptotic direction. TAp73 itself induces PIR2. PIR2 levels are upregulated during DNA damage but the changes in apoptotic response happen only with coexpression with TAp73 or ΔNp73. PIR2 seems to be a promising therapeutic target in controlling p73 isoform levels and improving chemosensitivity in tumor cells.

To target individual isoform and selectively silence these without having any lowering effect on the levels of other isoform is challenging. This has been successfully done *in vitro* with several different cell lines and i*n vivo* against melanoma by using modified antisense technology (Emmrich et al. 2009). The mRNA's of p73 NH_2- variants (ΔNp73 and ΔN'p73) were targeted in the intron-derived exon 3B, which is unique in both variants. Targeting was done with locked nucleid acid (LNA) antisense oligonucleotide (ASO) gapmers which the group of Emmrich et al. had developed. Magnetic nanobeads (MNBs) were used to optimize *in vivo* administration, as they prevent diffusion of the complex from the injection site. Specific therapeutic efficacy was improved by maintaining the ASO concentrated in tumor.

The results were the specific reduction in tumorigenic p73 transcripts and proteins (ΔNp73 and ΔN'p73) leading to balance towards apoptotic p73 variant (TAp73). The final response was decreased tumor cell proliferation and reduced tumor growth. Treatment with ASO's appear to be a new and successful way to target and control the balance of p73 isoforms in cancer cells.

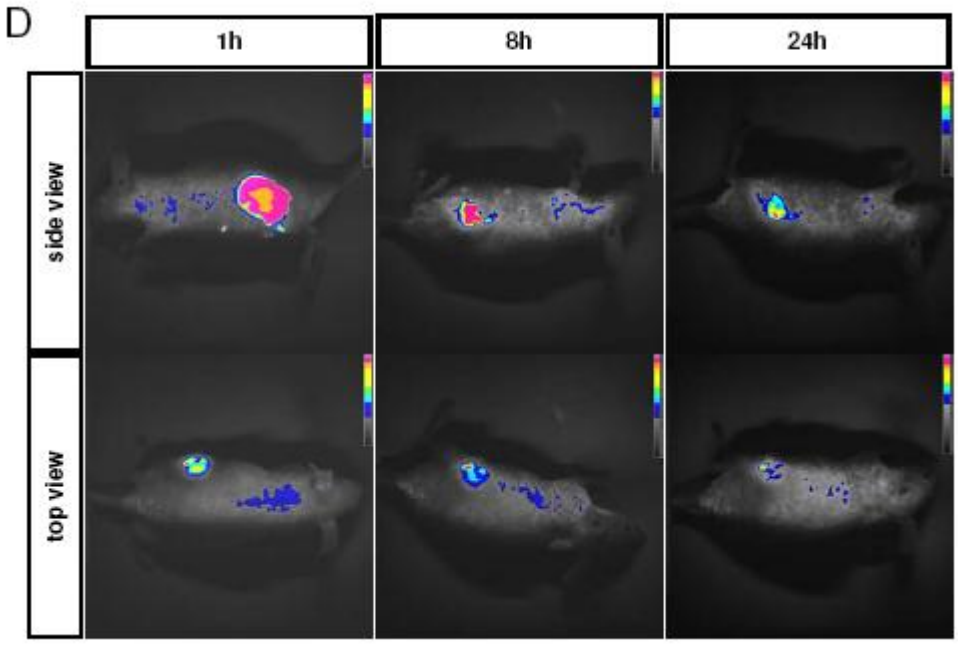


Table 5. *In vivo* fluorescence imaging of mice treated with ASO gapmers. Data was measured 1, 8 and 24 hours after intratumoral injection (Emmrich et al. 2009)

6 TARGET VALIDATION AND THERAPEUTIC APPROACHES

6.1 Target validation

Studies to evaluate the potential therapeutic targets of p73 are usually *in vitro* cancer cell-based assays. However, *in vivo* studies of p73 and its targets are more less frequent. What is seen in cells *in vitro* should also be seen in tumors *in vivo* to give a real estimate of the possible success of therapeutic approach. When considering the previously mentioned targets *in vivo*, ASPP1 and ASPP2 have been shown to bind to p53 family members *in vivo* and stimulate their apoptotic function (Bergamaschi et al. 2004). The inhibitory member iASPP's function has also been proven *in vivo* in tumors (Bergamaschi et al. 2003). Instead, the therapeutic approach towards iASPP has been tested only *in vitro* and the in vivo studies of upregulating the ASPP proteins could not be found. To estimate a possible successful therapeutic target, main issues which have to be considered are target's specificity, activity, risk of resistance and drugability. In coming chapters the *in vitro* and *in vivo* results of the main approaches towards p73 targets are introduced.

6.2 Itch knockdown and inhibition

In 2005, Rossi et al. demonstrated that the ubiquitin-protein ligase Itch regulates p73 stability during DNA damage and also in normal conditions (Rossi et al. 2005). Itch selectively binds and ubiquitinates p73 and p63 but does not have any effect on p53. During DNA damage Itch is downregulated and because of that, p73 is upregulated. Itch inhibition has been seen to regulate chemosensitivity of cancer cells *in vitro* (Hansen et al. 2007). The expression of Itch was knocked down by siRNA and shRNA (short hairpin interfering RNAs) in HeLa (cervical cancer) cells which increased basal expression of p73 and increased the number of apoptotic cells seen after genotoxic stress (e.g. cisplatin, doxorubicin). The apoptotic pathway was at least p73-mediated. p53-null cells seemed to be more sensitive to Itch-depletion than cells with functional p53. This finding points out the importance of Itch knockdown specifically in cells, that have absent or mutated p53 (almost half of the tumors). The shRNAs used were identical between human and mouse Itch.

Itch ligase connected to p73 has been getting attention in research and shows a good amount of *in vitro* studies but *in vivo* there are not many. One factor related to p73 and Itch also *in*

vivo studies is a protein interactor and Nedd4-binding partner-1 (N4BP1). N4BP1 is a substrate of Itch ligase and these two structurally related proteins have a functional interaction with each other (Oberst 2007). N4BP1 binds to the second WW domain of Itch and interferes Itch from binding to its substrates. N4BP1 and p73α have the same binding site to Itch and by binding to Itch N4BP1 prevents the Itch-mediated ubiquitination of p73, seen *in vitro* and also *in vivo* in ubiquitylation assays. The role of N4BP1 in tumor progression and in response of cancer cells to chemotherapy seems evident.

MicroRNAs (miRNAs) are small RNAs that regulate gene expression at a posttranscriptional level by affecting negatively to a target mRNA (messenger RNA) (Ambros et al. 2003). They inhibit translation of mRNA by inaccurate antisense base-pairing, the mechanism making them different from siRNAs. MicroRNAs are the same size as siRNAs but siRNAs are more precise and cause RNA-mediated interference.

MicroRNAs have also been targeted Itch to achieve its degradation by microRNA 106b (Sampath et al. 2009). The study was done in several cancer cell lines, H1299, HeLa, K562 and primary leukemia cells. The cells were exposured to deacetylase inhibitors, which was seen to result in transcriptional induction of miRNA 106b (Figure 5). The induction of previously mentioned miRNA was connected to the downregulation of Itch levels and to enhanced p73-mediated apoptosis. The underlying mechanism was shown to be the acetylation of histones around the Mcm7-miR106b promoter caused by deacetylase inhibitors. This led to enrollment of e.g. E2F1 to the Mcm7-miR106b promoter and together with host gene Mcm7 caused transcriptional induction of miR106b. The p73 up-regulation was associated e.g. with p73 target PUMA causing mitochondrial dysfunction, leading to cell death.

The chemotherapeutics drugs that activate miR106b should be studied more because miRNA may be a possible new mechanism to target Itch in cancer cells. The characterization of cancers with low miR106b expression could provide answers to weak apoptotic responses. The study also revealed a small group of samples that did not show any induction of miRNA in response to deacetylase inhibition, suggesting an alternative pathway regulating Itch.

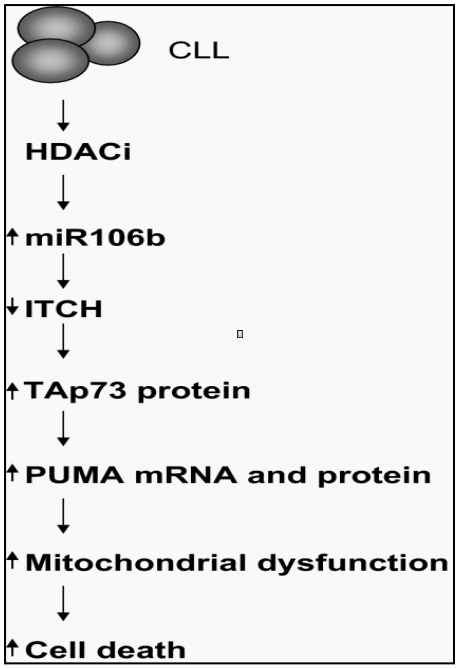


Figure 6. A pathway of deacetylase inhibitor-mediated miRNA induction, leading to Itch down regulation and upregulation of Tap73 levels. The final result is mitochondrial dysfunction and enhanced cell death, results seen in chronic lymphotic leukemia cells (Sampath et al. 2009).

6.3 Nutlin-3

HDM2 (human double minute oncogene) is a negative regulator of p53 and p73 but which does not induce degradation of p73 (Balint, Bates & Vousden 1999). HDM2 binds to p73 and suppresses its transcriptional activity. Nutlin-3 instead is a small molecule (molecular weight 581.5 g/mol), which inhibits the interaction between p73 isoform TAp73 and HDM2 in p53-null or p53-mutant cells (Lau et al. 2008). The inhibition of binding between p73 and HDM2 leads to increased p73 transcriptional activity at protein level and to enhanced apoptosis mediated by p73 downstream genes e.g. noxa and PUMA. In the study by Lau et al, Nutlin-3 treatment resulted to *in vitro* dose-dependent inhibition of growth in neuroblastoma, colon-carcinoma and osteosarcoma cells.

The optimal dose of Nutlin-3 was 20-30 µM, being able to interrupt the p73-HDM2 complex formation and leading to activation of p73 and its targets. Therapeutic use of Nutlin-3 could provide a mechanism to increase p73-mediated apoptosis in cancer cells and improve responses to chemotherapeutic treatment. *In vitro* assays have proven Nutlin's effect in both, wild-type p53 and mutant p53 cells (Lau et al. 2008) ; (Ray 2011) ; (Ambrosini et al. 2007). *In vivo* assays with Nutlin-3 in xenograft tumor models had shown potent effects but only the

p53 function has been under investigation (Endo et al. 2011). The connection between p53-family members is clear but the specific role of p73 in cancer treatment with Nutlin-3 has not been evaluated.

6.4 Adenovirus- and PPIG3-DNA -mediated transfer of p73

An adenovirus-mediated transfer of p73 has been used to induce cell cycle arrest and apoptosis in human colorectal cancer cell lines *in vitro* and *in vivo* in mice (Sasaki et al. 2001). Overexpressed exogenous p73 and p63 were able to activate also p53 target genes and cause cell death. In the study by Sasaki et al. tumor growth in mice injected with adenovirus-mediated p73 was reduced, compared to controls which had normal levels of p73. By comparison the effect seen with adenovirus-mediated transfer of p53 was weaker than with p73. When p73 subtypes were compared, p73β was found to be more than p73α. The adenoviral vector is considered suitable for gene therapy in clinical use because it has broad host range, high transduction efficiency and the production of purified vector can be easily scaled up for larger production, even though problems have also been associated expecially with its use as systematic injection (off-target effects and innate-immune responses) (Couto, High 2010).

Adenovirus expressing p73β has shown promising results also in the treatment of cervical cancer *in vivo*. The adenovirus based p73 gene therapy targeted to cervical cancer inhibited the tumor growth of already established tumors in mice (Das 2006). The human xenografts in nude mice were used in the study. Tumor growth inhibition was due to the stable expression of p73 protein together with the simultaneous induction of p73 target gene p21. The importance of E2F1 gene therapy could offer a potential approach expecially in cervical cancer, because the HPV oncoprotein E6 inactivates p53 protein by targeting and ubiquitinating it. This causes the p53 based gene therapy to be more prone to failure.

Adenovirus-mediated TAp73β gene transfer leading to overexpression has been shown to also overcome the chemotherapeutic drug resistance *in vitro* in human malignant melanoma, caused partly by the dominant-negative form of p73 (ΔNp73) (Tuve 2006). TAp73β sensitized 5 of 7 melanoma cell lines to treatment with adriamycin and cisplatin. In xenograft mouse models, the overexpressed TAp73β, together with a genotoxic drug, inhibited melanoma cell growth. In clinical studies, adenovirus-mediated transfer of p53 in 82 patients

with hepatocellular carcinoma showed promising results in one-year follow-up study (Guan et al. 2011). After 12 months the survival rates in p53 gene therapy treated group were almost 50% better than in control group. Both groups received TACE (transcatheter hepatic arterial chemoembolization) as a basic treatment. Pain and side-effects were also observed less in the p53 gene therapy treated group. This result indicates a possible success for p73-based gene therapy in future cancer treatment.

A factor in p73 pathway, E2F1 has also been shown to enhance antitumor activity *in vivo* when used in adenovirus-mediated gene transfer (Dong et al. 2002). In melanoma, the tumor growth decreased from 87 to 91 per cent (compared with controls) in nude mice when the animals were treated with anticancer drugs combined with E2F1-based gene therapy. In pancreatic cancer, the E2F1 adenovirus-mediated gene transfer has also shown similar results (Rodicker et al. 2001). The effect of E2F1-based gene therapy has been associated with the induction of p73, highlighting the potential use of E2F1/p73 pathway in cancer therapy.

PPIG3/DNA (polypropylenimine dendrimer) nanoparticles in gene transfer have shown good results in *in vitro* but also *in vivo* in mice (Chisholm et al. 2009). Nanoparticles are proven to specifically target and accumulate in tumors after systemic injection, without having any transferring effect on the surrounding tissues. The nanoparticles are in a form of well-organized fingerprint-like structure, having DNA molecules inside. The colloidal stability is an important quality of nanoparticles, attained in the study of Chisholm et al. Also the expression of transgene in tumor had succeeded, testifying the potential use of PPIG3 nanoparticles in cancer gene therapy and possibly as a tool for upregulating p73.

6.5 p73 isoforms

The importance of targeting towards specific isoforms of p73 has *in vitro* but also *in vivo* evidence, especially to the dominant-negative isoform ΔNp73. Transgenic mice expressing ΔNp73 in the liver showed hepatic histological abnormalities, for example increased hepatocyte proliferation causing liver cell adenomas in 3-4 months (Tannapfel et al. 2008). After 12 to 20 months the 83 per cent of mice had developed hepatic carcinoma. This proves the independent and crucial role of ΔNp73 in tumor development, making it a possible target for cancer treatment and a marker in cancer diagnosis. It is also proven to cooperate with Ras

oncogene, causing *in vivo* the development of fibrosarcomas in nude mice (Petrenko, Zaika & Moll 2003).

7 CONCLUSIONS

The p53-family of tumor suppressors have important functions related to cell cycle regulation, development and tumorigenesis, especially they are studied for functions concerning cell cycle arrest and apoptosis. p53 is found to be mutated in cancer but p73 mutations are rare (Kaghad et al. 1997). It is important to maintain the tumor suppressor function also in cells having mutated p53 and p73 is able to take the role as a main tumor suppressor in cells having mutated p53 (Willis et al. 2003). Rare p73 mutations in cancer cells make it a more viable target for anticancer therapy. p73 is regulated by several factors, making the approaches towards it complex (Tozluoğlu et al. 2008).

The levels of p53-family members are upregulated during DNA damage caused by e.g. anticancer agents or irradiation (Muller et al. 2006). Blocking the function of those tumor suppressors leads to chemoresistance (Seitz et al. 2010). Instead, the approaches to upregulate the p53 and p73 levels and make cancer cells more chemosensitive have been studied and may become a future therapy for the treatment of cancer. One promising target is Itch ligase, the negative-regulator of p73. Itch is connected e.g. to Runx and YAP1 but the connection is only recently studied and the consequences of Itch regulation to those factors should be studied more (Levy, Reuven & Shaul 2008). Itch has been also connected at least to Nedd4 member N4BP1 and MDM2 (Kubo et al. 2010); (Oberst 2007). Itch regulation is a fairly new field of research and the amount of *in vivo* studies is small. More studies should be done to evaluate its possible role in cancer treatment. Luckily successful *in vitro* studies give a good stepping stone for *in vivo* research and towards more efficient clinical trials.

Adenovirus-mediated transfer of p73 has shown good results *in vitro* but also *in vivo* (Tuve 2006) but the possible problems connected to virus-mediated gene therapy (innate-immune responses, off-target effects) may become problematic in clinical treatment (Couto, High 2010). PPIG3-DNA dendrimer nanoparticles have been seen as a more safe option for future gene therapies (Chisholm et al. 2009). They could be used as direct p73 gene transfer or as indirect e.g. in siRNA/shRNA-mediated Itch knockdown. Targeting towards specific p73 isoforms (transactivating TA or dominant-negative ΔN isoforms) could also be effective way to enhance apoptosis and cell cycle arrest in cancer cells, if only a validated way of isoform

regulation could be found. The network surrounding p73 is extensive, consisting of several regulators and inhibitors (Tozluoğlu et al. 2008). This indicates that there is a considerable number of possible therapeutic targets, which may offer options for future cancer therapy.

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EXPERIMENTAL PART: THE ROLE OF P73 AND ITCH IN PANCREATIC CANCER CELLS

TABLE OF CONTENTS

1 INTRODUCTION

p73 is a member of p53 family of tumor suppressors, consisting of p53, p63 and p73 (Kaghad et al. 1997). The p53-family members are connected to several functions in cell cycle regulation, development and tumorigenesis. p73 gene encodes a protein with similar characteristics of p53 and p63. The family members have similar but also individual roles (Levrero et al. 2000). p73 is involved in apoptosis and cell cycle arrest in cancer cells. p53 protects against the proliferation of damaged cells and this way decreases tumour growth. Genetic mutations of p53 appear in half of the human cancers, resulting as loss or inactivation of p53 (Hollstein et al. 1994). The extensive amount of mutations in p53 make therapeutic approach towards it difficult. Instead, p73 has been found to be more rarely mutated in cancers than p53 and is considered to be more viable target for anticancer therapy approach (Kaghad et al. 1997).

One major factor regulating the levels of p63 and p73 is ubiquitin-protein isopeptide (E3) ligase Itch. Itch selectively binds to the protein's PY-motif and ubiquitinates it, causing its rapid proteosomal degradation (Rossi 2006) ; (Rossi et al. 2005). In ubiquitination, the protein is inactivated by covalent attachment by one or more ubiquitin monomers. p53 does not have PY-motif and it is negatively controlled by MDM2, not by Itch. Controlled knockdown of Itch by siRNA/shRNA leading to the induction of p73 levels could offer a potential therapeutic method to improve cancer treatment in the future.

2 THE AIM OF THE STUDY

The work was carried out between October 2008 and February 2009 in School of Pharmacy, University of London. It was done as a part of a bigger research project in which the main goal was to use a dendrimer nanoparticle to deliver siRNA to pancreatic cancer cells, aiming to down regulate Itch and as a result cancer cells to become more sensitive to anticancer drugs mediated by p73 upregulation.

The experimental part included basic cell culture, optimizing the conditions for cancer cell transfection using dendrimer-complexes, measurement of cytotoxicity of the complexes, characterization of pancreatic cancer cell lines and measurement of Itch and p73 protein levels in pancreatic cancer cell lines. The cell lines used were B16F10 (melanoma cancer), A431

(epidermoid cancer), MDAMB 231 (breast cancer) and 4 different pancreatic cancer cells PANC1, BxPc-3, Mia PaCa-2 and HPAC. In protein extraction also HeLa (cervical cancer) and H1299 (non-small cell lung carcinoma) cell lines were used as a comparison material.

Similar studies have not been done before in pancreatic cancer cell lines. Itch downregulation with siRNA has been tested in vitro by Hansen et al. in 2007, where it was combined with chemotherapeutic drugs and led to enhanced p53-family mediated apoptosis in HeLa cells (Hansen et al. 2007). The study also showed that cells with no functional p53 (mutated or absent) were more sensitive to Itch down regulation, proving the role of Itch in majority of cancer having mutated p53.

3 PANCREATIC CANCER AS A TARGET FOR EFFECTIVE CANCER THERAPY

Pancreatic cancer is a severe disease and usually a patient is confronted with a poor prognosis, the overall 5-year survival rate in all pancreatic cancer stages being 5 to 6 per cent (Jemal et al. 2010). In a recent phase 2 trial by Mamon et al. patients with non-metastasive pancreatic cancer treated with gemcitabine, 5-fluorouracil and radiation therapy had overall survival rate of only 12 months (follow-up time 55 months) (Mamon et al. 2011). An objective tumor response was seen in 25 per cent of the patients. In USA from 1990 to 2006 the death rates have grown for women having pancreatic cancer (Jemal et al. 2010). Early detection and screening of the disease is reasonable only in high risk patients (Xu, Zhang & Zhao 2011). Gemcitabine is used as a standard treatment of pancreatic cancer and radiotherapy is under development towards more modern radiation techniques, hopefully estimating better results in the future. The failure of the therapy in pancreatic cancer has been proposed to stem from the heterogeneous molecular pathogenesis of pancreatic cancers, involving several oncogenic pathways and mutations (Li, Saif 2009). More profound cancer treatments are needed, directing the research to explore new molecular targets and gene therapy.

Gene therapy by adenovirus-mediated p73 upregulation has shown promising results in vitro in p53-null pancreatic cancer cells and supports the use of p73 in cancer treatment (Rodicker, Putzer 2003). In vivo studies specifying the role of p73 in adenovirus-mediated gene therapy in pancreatic cancer have not been done so far and safety issues are associated with the use of viral vectors e.g. in RNA interference (off-target effects, innate immune responses) (Couto, High 2010).

Instead, the use of PPIG3/DNA (polypropylenimine dendrimer) nanoparticles in gene transfer has shown good results in in vitro but also in vivo in mice (Chisholm et al. 2009). Nanoparticles are proven to specifically target and accumulate in tumors after systemic injection, without having any transferring effect on the surrounding tissues. The nanoparticles are in a form of well-organized fingerprint-like structure, having DNA molecules inside. The colloidal stability is an important quality of nanoparticles, attained in the study of Chisholm et al. Also the expression of transgene in tumor had succeeded, testifying the potential use of PPIG3 nanoparticles in cancer gene therapy.

4 MATERIALS AND METHODS

4.1. Transfection of B16F10 cell line with DAB16 dendrimers

Melanoma cancer cell line B16F10 was used for evaluating the most efficient amount of DAB16-dendrimer complex for the transfection assays. The ratio of N/P8 and N/P30 was used and the complex amounts were 0,5 µg, 1 µg, 2 µg, 5 µg and 10 µg. 10 000 cancer cells per well were seeded to a 96-well plate and the cells were grown for 3 days in supplemented medium suitable for B16F10 cells (DMEM, FBS, L-glutamin). Complexes were made by the protocol (Appendix 2**)** and the dilution of the complex was in 1/10 ratio to 5% dextrose when needed. Before the transfection, the cells were washed with DPBS. Complexes were added to the cells and the 100 µl of medium (DMEM + 10 % FBS) was added to each well. Lipofectamin was a positive control of the transfection. Incubation time for the plates was 4 hours at 37 degrees (CO_2 5 %*)*. After the incubation cells were cleared from the complexes and medium and supplemented medium was added. Cells were grown for 2 days and analysed by β-galactosidase according to the protocol (Appendix 3)

4.2. Size measurement of the complex (DAB16 N/P30)

Size of the complex (DAB16 N/P30 + pDNA) was measured in two different mediums to optimize the transfection conditions. The mediums were DMEM + 10 % FBS and plain DMEM. The size of the complex was examined also without any medium. The complexes were made by the protocol (Appendix 2**)** and HEPES was used as a complex buffer. The measurements were done by

Zetasizer. The first measurement took place 15 minutes after complex formation. Then 200 µl of complex was added to 500 µl of both mediums and incubated for 4 hours in 37 degrees *(*CO_2 5%). After incubation the second measurement was done.

4.3. Transfection efficacy with different buffers and mediums to cell lines A431 and MDAMB 231

The assay was done to evaluate the differences in transfection efficacy when the ratio of DAB16 dendrimer (ratios N/P8 and N/P30) and the complex buffers and transfection mediums were changed (buffers HEPES and MES, mediums plain DMEM and DMEM with 10% FBS). Complexes and transfection were done by the protocols (Appendix 2 and 3) and after the tranfection the plates were incubated for 4 hours in 37 degrees (CO_2 5%). After incubation the transfection medium was removed and the cells were washed with DPBS. The suitable medium for the both cell lines (Appendix 1) was added and after 2 days the transfection efficacy was analysed by β-galactosidase (Appendix 3).

4.4. Transfection and cytotoxicity assays in pancreatic cancer cells

Transfection assay was done to optimize the time points after transfection and compare it with the cytotoxicity results. Pancreatic cancer cells (Mia PaCa-2, PANC1, HPAC and BxPc-3) were seeded 1000 cells/well and grown for 3 days in 37 degrees (CO_2 5%). Cells were transfected by the protocol with pDNA-dendrimer (N/P30) complex, the amount used was 1 µg (Appendix 3). Transfection medium was DMEM with 10 % FBS. Lipofectamin was a positive control. After incubation the cells were cleared from the medium, washed with DPBS and completed with every cell line's specific medium (Appendix 1) Cells were grown from 1 day to 4 days. At different time points the transfection efficacy was analysed by β-galactosidase analysis (Appendix 3)

Cytotoxicity assay's aim was to give an explanation of what happens to cell viability after exposing cells to complexes and plain dendrimers and possibly for the recovering of the cells in different time points after transfection. All four pancreatic cancer cell lines were seeded 1000 cells/well and grown for 3 days in 37 degrees (CO_2 5%**)**. After 3 days, plain DAB16 dendrimer solution (maximum concentration 20 mg/ml) or DAB16 N/P30 complexes (maximum concentration 0,25

mg/ml) were added to the cells. Cells were incubated for 4 hours in 37 degrees (CO_2 5%). After incubation the cells were cleared from the solutions and washed with DPBS. Cytotoxicity of the complexes and plain dendrimers was measured by MTT-assay (Appendix 3)

4.5. Protein level measurements of p73 and Itch

Measurement of protein levels was done by Western blotting by the procol (Appendix 5). α-actin was used as a positive protein control. Primary antibodies were p73 clone ER-15 (Ab-2 ER15 mouse monoclonal antibody, Thermo Scientific) and Itch (purified mouse anti-Itch Mab, BD Transduction Laboratories). Dilutions for antibodies were done according to the provider's instructions, p73 diluted 1:100, Itch 1:1000 and α-actin 1:500. The second antibody (goat anti-mouse IgG (H+L) horseradish peroxidase conjugate) was diluted 1:5000.

5 RESULTS

5.1. DAB16 transfection of B16F10 cell line

Results showed that N/P30 transfects better than N/P8 in B16F10 cell line (Figure 1). Most efficient amount of complex for transfection was 1 ug. There are missing few results of N/P8, because results were out by β-galactosidase-analysis and gave minus values.

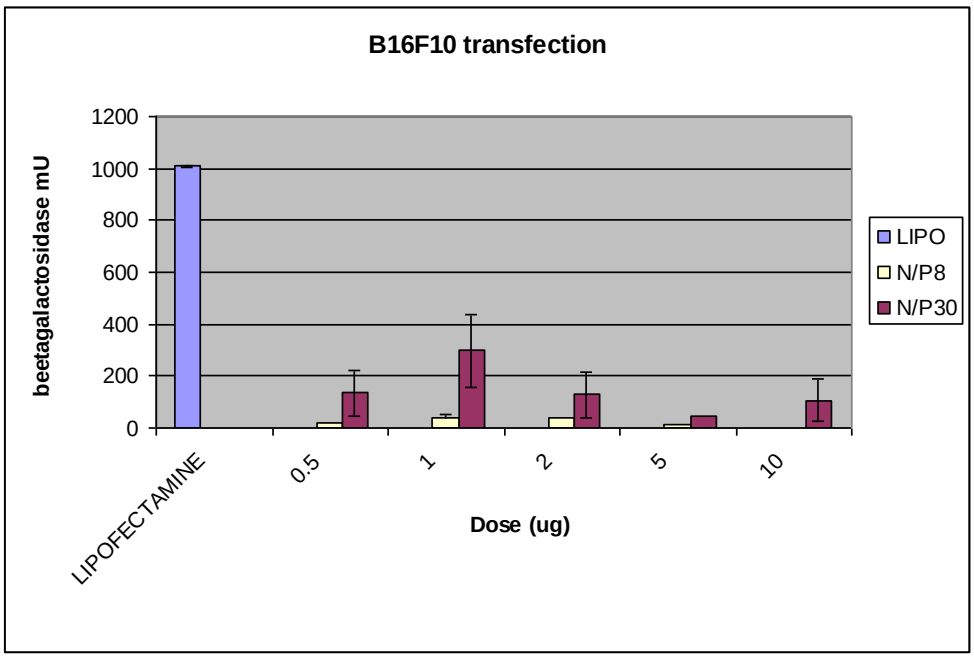

Figure 7. Transfection of B16F10 cells with DAB16 N/P8 and N/P30 complex. The amount of the complex used was 0,5 µg, 1 µg, 2 µg, 5 µg and 10 µg. Lipofectamin was used as a control and the transfection efficacy was measured by β-galactosidase analysis.

5.2. Size of the complex

To evaluate the effect of the complex size in transfection, transfection was done in DMEM + 10% FBS and in plain DMEM. As seen in Table 1, the complex stays smaller in medium of DMEM + 10 % FBS than in plain DMEM.

Table 2. The average size of the complex DAB16 N/P30 without medium, in mediums DMEM + 10% FBS and plain DMEM. Standard deviations of the results are shown on the right side of the table.

	Size (average)	**+/-**
Complex	**226.2**	**2.2**
Complex in DMEM + 10% FBS	**394.8**	**5.5**
Complex in plain DMEM	**4310.6**	**398.9**

5.3. Comparison of the transfection efficacy with different buffers and mediums

With cell line MDAMB 231 mistake was made (N/P30 in HEPES, DMEM with 10% FBS) and the results were not reliable (data not shown). Overall it was seen from the results (Figure 2 and 3**)** that transfection is more efficient with complex buffer HEPES in the medium of DMEM + 10% FBS than with buffer MES. Transfection efficacy was lower in DMEM with 10% FBS than in plain DMEM. In plain DMEM the complex was bigger (Table 1), so we could assume that using HEPES is better in complexes and DMEM with 10% FBS keeps the size of the complex smaller. N/P30 seemed to be more effective in transfection than N/P8. Differences in transfection between the cell lines were seen, in average demonstrating better transfection in A431 cell line.

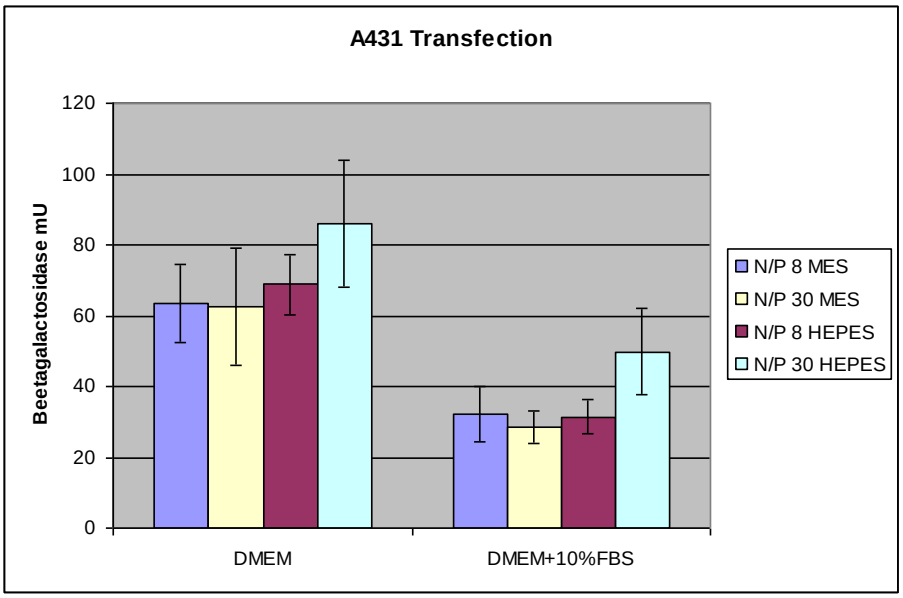

Figure 2. Transfection of A431 cell line with DAB16 N/P8 and N/P30. DMEM + 10% FBS and plain DMEM were used as transfection mediums and complex buffer was HEPES and MES. Transfection efficacy was measured by β-galactosidase analysis.

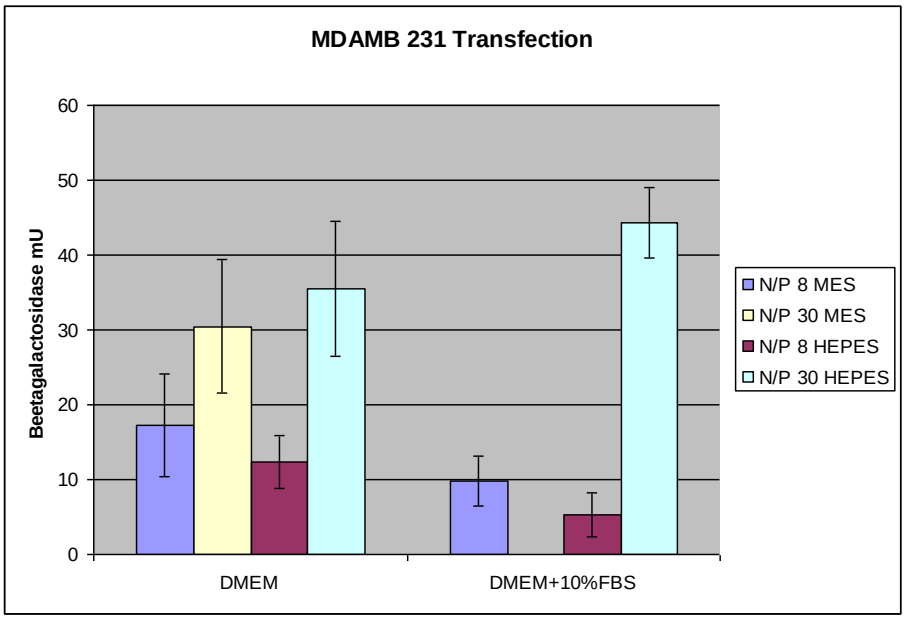

Figure 3. Transfection of MDAMB 231 cell line with DAB16 N/P8 and N/P30. DMEM + 10% FBS and plain DMEM were used as transfection mediums and complex buffer was HEPES and MES. Transfection efficacy was measured by β-galactosidase analysis

5.4. Transfection and cytotoxicity of DAB16 N/P30 in pancreatic cancer cells

Results seen in Figure 4 show, that DAB16 N/P30 transfection succeeded most after 2-3 days. Cell line PANC1 was transfected the most and differences between the transfection of the cell lines were small (except BxPc-3). BxPc-3 cell line didn't show any transfection after 1 day and even Lipofectamin wasn't able to transfect the cell line (Figure 5). After 4 days of transfection, the results started to show bigger error bars and results were not reliable.

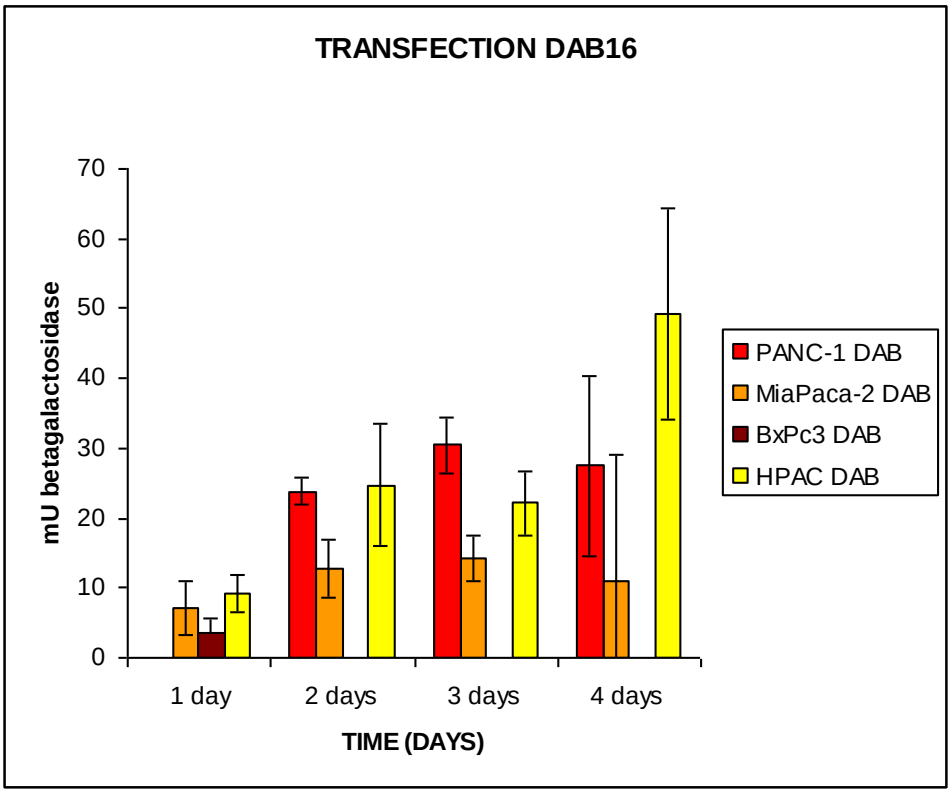

Figure 4. Transfection efficacy of pancreatic cancer cell lines with DAB16 N/P30 after 1-4 days. Measurement was done by β-galactosidase analysis.

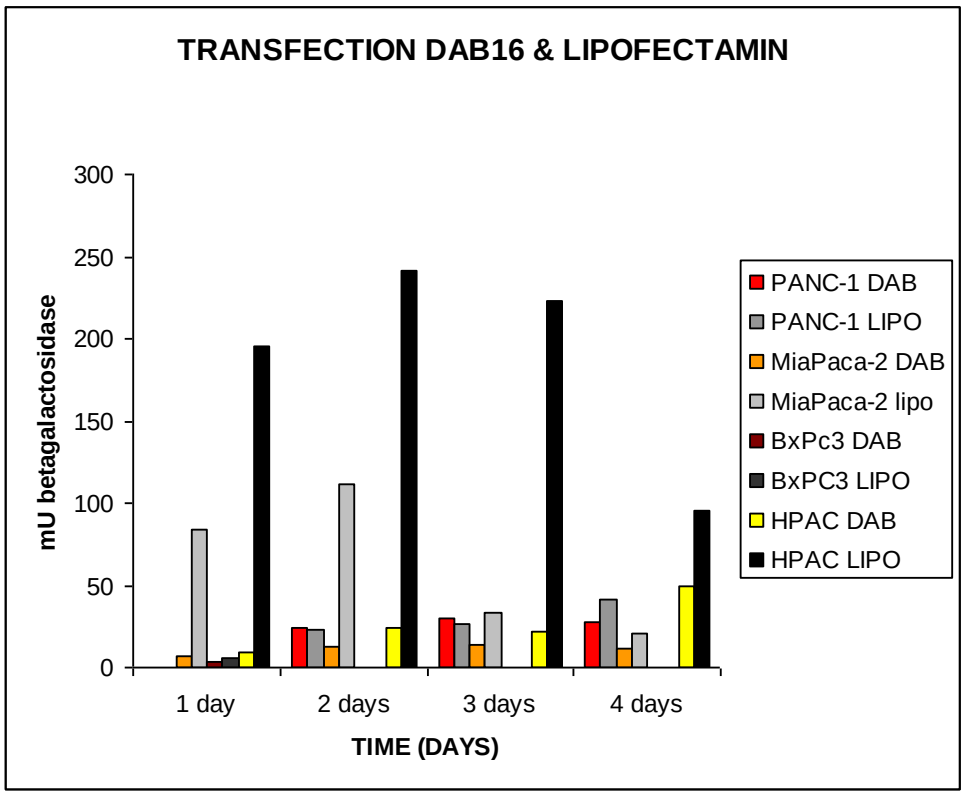

Figure 5. Transfection efficacy of pancreatic cancer cell lines with DAB16 N/P30 after 1-4 days. Measurement was done by β-galactosidase analysis. Lipofectamin was used as a control.

As seen in transfection assays **(**Figures 4 and 5**)** cell line BxPc-3 was almost impossible to transfect and the transfection efficacy was poor. On the contrary, the cytotoxity assays showed similar results for BxPc-3 as for other cell lines (Figures 8 and 9) indicating the possible existence of the mechanisms in BxPc-3 cells which differ from other pancreatic cancer cells and interfere complexe's way to the nucleus. Results of the PANC1 and HPAC didn't succeed as well as the others, showing more standard deviation in the values (Figures 11 and 13). Specific results of the cytotoxicity for each cell line are seen in Figures 6-13. IC50-values (Table 2) show recovering of the cell survival in 3 days post-incubation compared to 1 day post-incubation values.

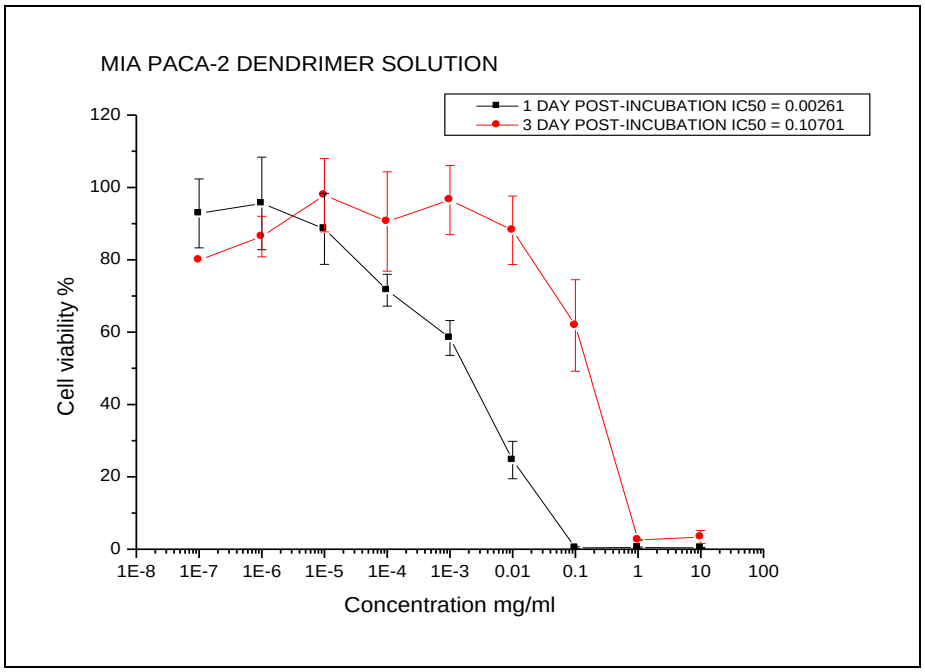

Figure 6. Cell viability (%) and IC50 values of Mia PaCa-3 cells after the dendrimer solution (concentrations mg/ml) was added (measured the first (black line) and third (red line) day). Cell viability was analysed by MTT-assay.

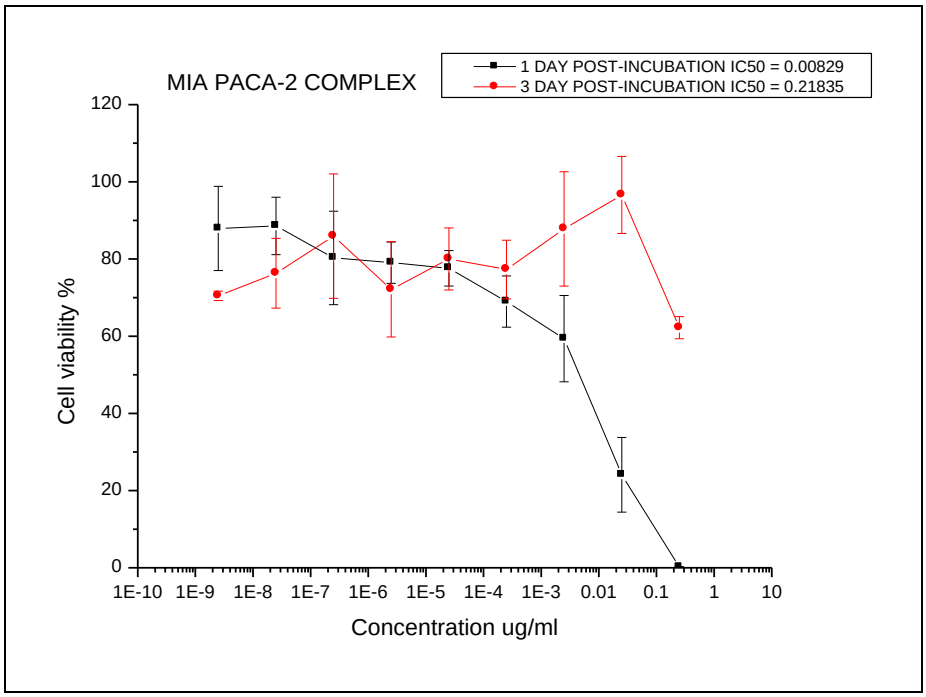

Figure 7. Cell viability (%) and IC50 values of Mia PaCa-3 cells after the complex solution (concentrations mg/ml (not ug/ml)) was added and measured the first (black line) and third (red line) day. Cell viability was analysed by MTT-assay.

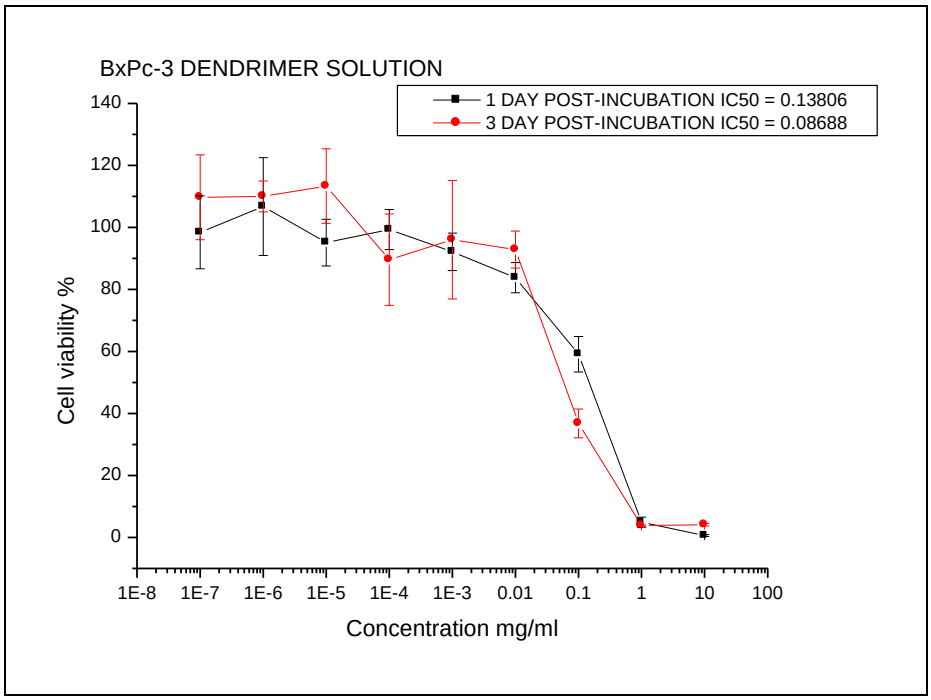

Figure 8. Cell viability (%) and IC50 values of BxPc-3 cells after the dendrimer solution (concentrations mg/ml) was added and measured the first (black line) and third (red line) day. Cell viability was analysed by MTT-assay.

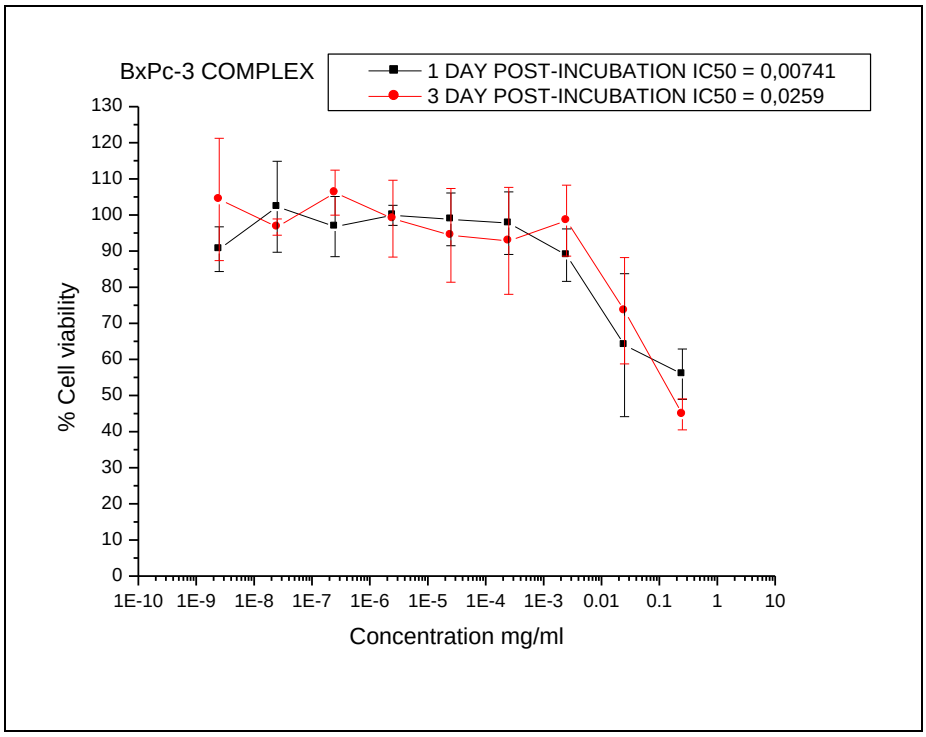

Figure 9. Cell viability (%) and IC50 values of BxPc-3 cells after the complex solution (concentrations mg/ml) was added and measured the first (black line) and third (red line) day. Cell viability was analysed by MTT-assay.

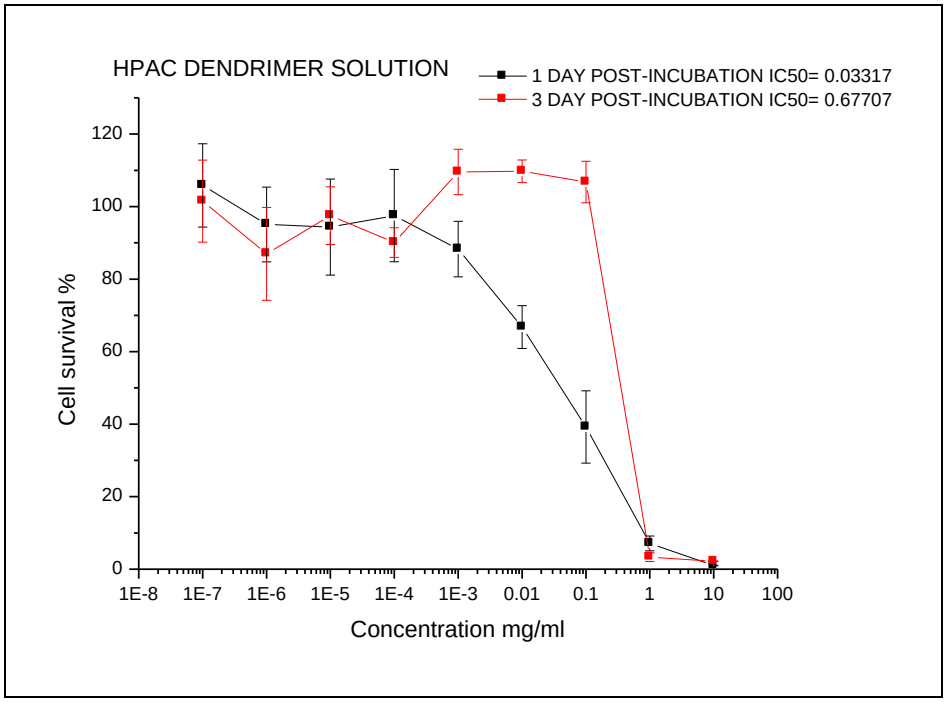

Figure 10. Cell viability (%) and IC50 values of HPAC cells after the dendrimer solution (concentrations mg/ml) was added and measured the first (black line) and third (red line) day. Cell viability was analysed by MTT-assay.

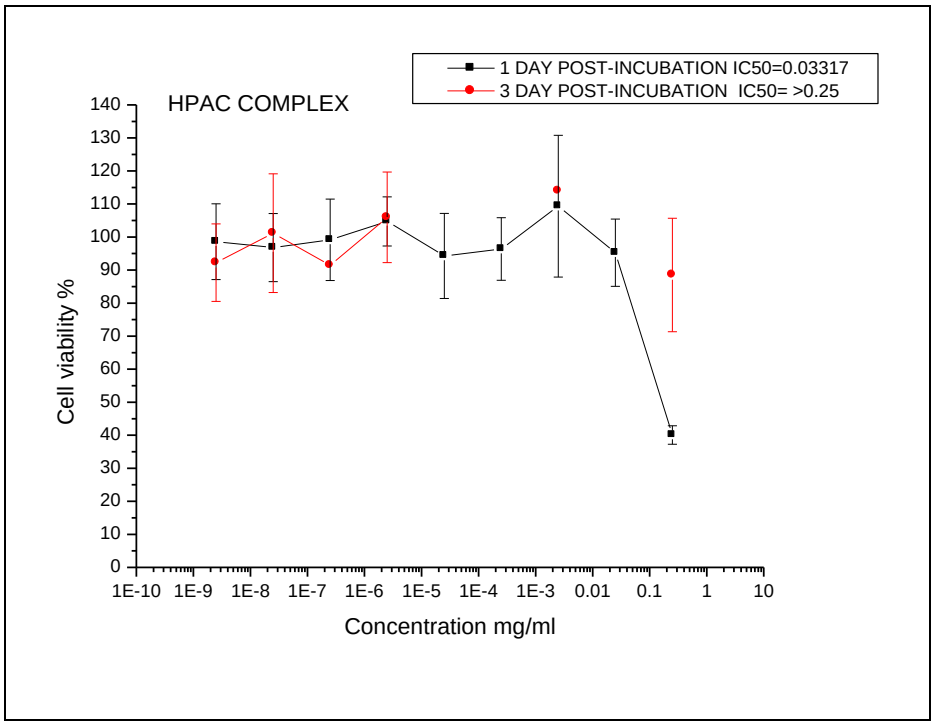

Figure 11. Cell viability (%) and IC50 values of HPAC cells after the complex solution (concentrations mg/ml) was added and measured the first (black line) and third (red line) day. Cell viability was analysed by MTT-assay. (Some values of 3 day post-incubation could not be measured by MTT-assay and are missing from the figure)

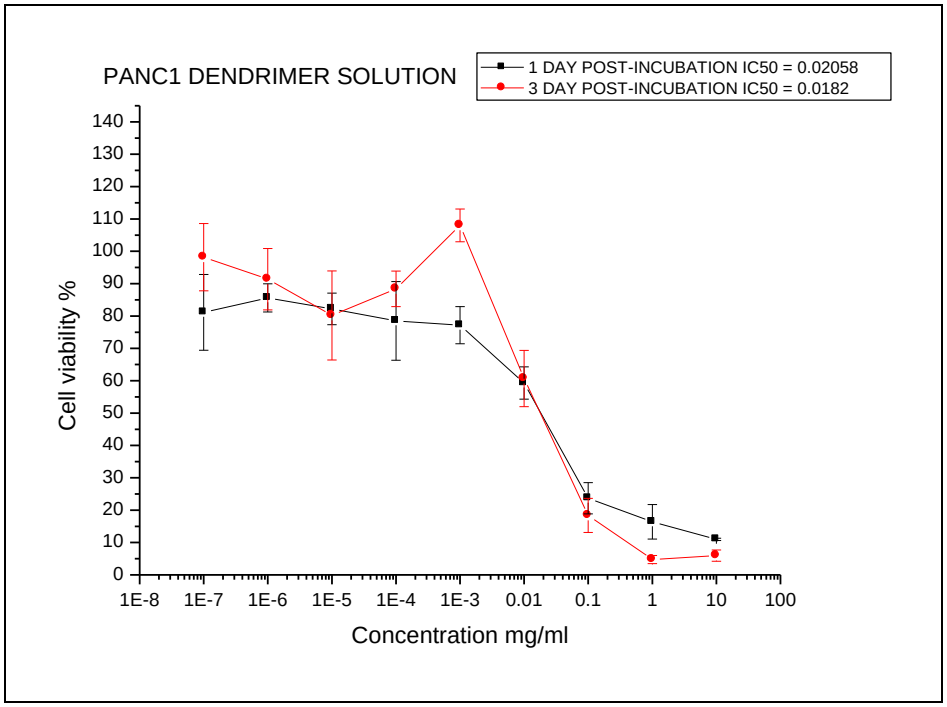

Figure 12. Cell viability (%) and IC50 values of PANC1 cells after the dendrimer solution (concentrations mg/ml) was added and measured the first (black line) and third (red line) day. Cell viability was analysed by MTT-assay.

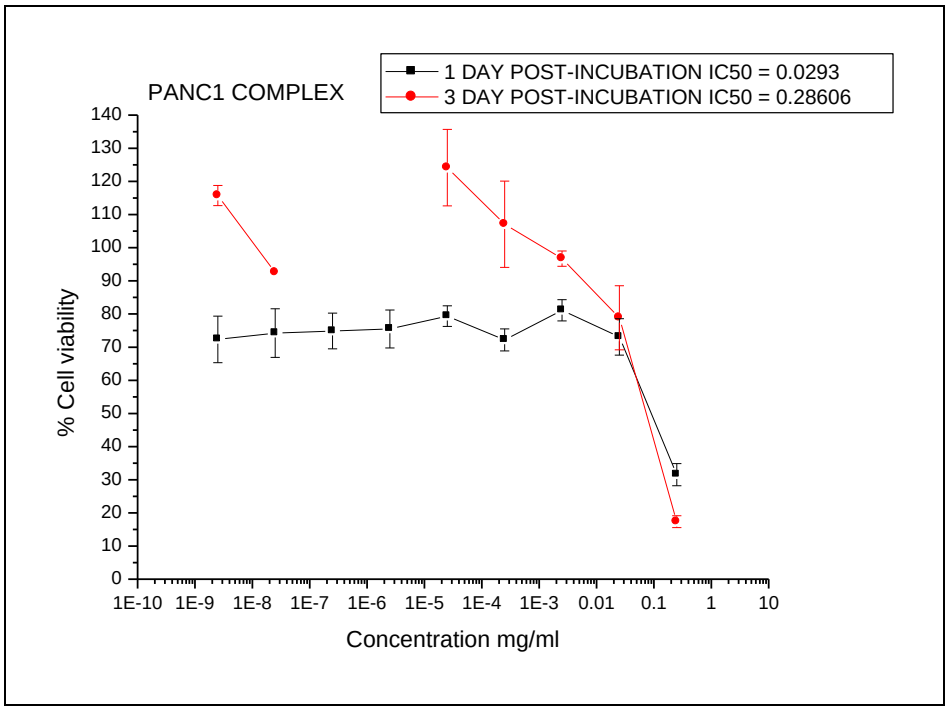

Figure 13. Cell viability (%) and IC50 values of PANC1 cells after the complex solution (concentrations mg/ml) was added and measured the first (black line) and third (red line) day. Cell viability was analysed by MTT-assay. (Some of the values of 3 day post-incubation were out in MTT-assay)

Table 3. IC50-values (the half maximal inhibitory concentration) of the pancreatic cancer cell lines exposed to DAB16 N/P30 dendrimer solution or N/P30 + pDNA complex solution. The results show recovering of the cell survival in 3 days post-incubation compared to 1 day post-incubation values.

Cell line		Complex 1 day post-incubation	Complex 3 days post-incubation	Dendrimer 1 day post-incubation	Dendrimer 3 days post-incubation
PANC1		0.0293	0.28606	0.02058	0.0182
Mia PaCa		0.00829	0.21835	0.00261	0.10701
HPAC		0.03137	>0.25	0.03317	0.67707
BxPc-3		0.00741	0.0259	0.13806	0.08688

5.5. Western blot for Itch and p73

The molecular weight marker left some bands out so analyzing the weight was not optimal. α- actin (42 kDa) (Figure 14) was near 30-40 kDa. p73 (70-80 kDa) (Figure 15) didn't give any results and Itch (113 kDa) (Figure 16) showed good bands, though unspesific ones.

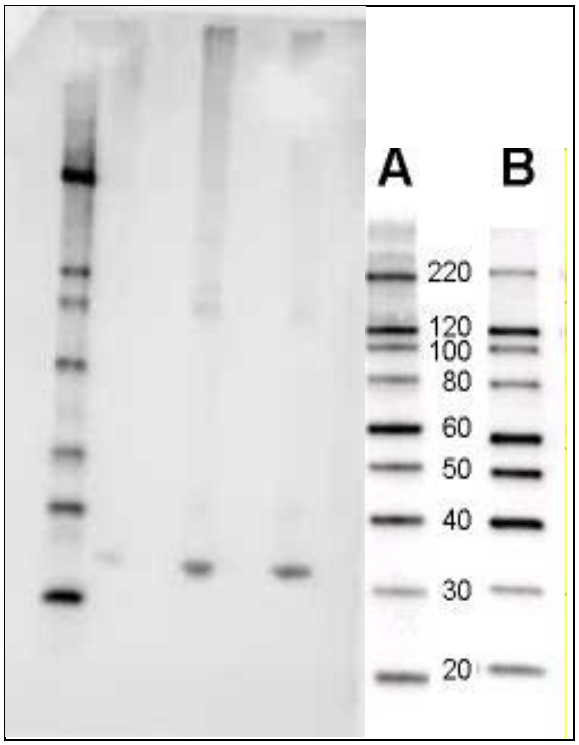

Figure 14. Westen blot results of a control protein α-actin
42 kDa. Proteins from cells Mia PaCa-2 (next to the ladder), PANC1 and HPAC.

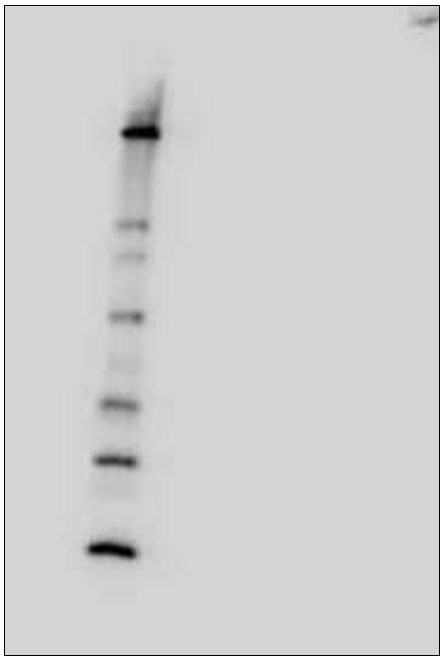

Figure 15. Western blot for p73 (70-80 kDa).
Only the ladder was seen in the results.

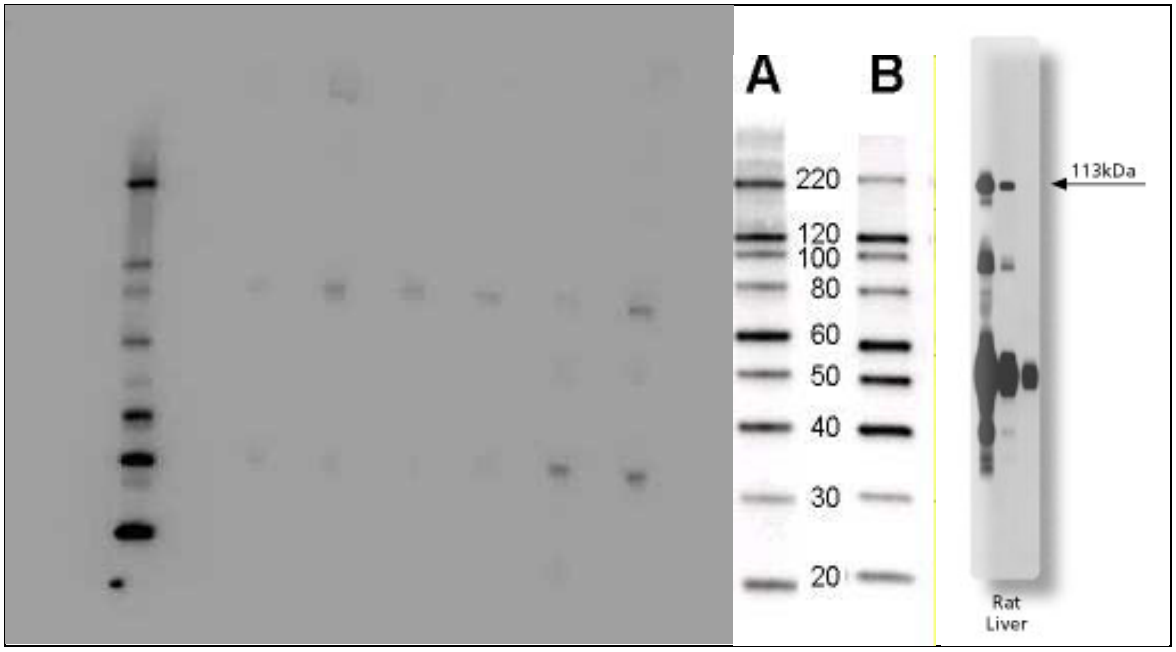

Figure 16. Western blot result for Itch protein (113 kDa). Proteins (in order from left to right next to the ladder) Mia PaCa-2, PANC1, HPAC, BxPc-3 HeLa and H1299. Only unspecific bands were seen. The picture on the right side is a WB of Itch taken from the antibody's provider (BD Transduction Laboratories). Row in the middle is the Itch-antibody dilution 1:1000, which was also used in the work.

6 DISCUSSION

The main focus of the work was to optimize the conditions for the transfection (complex amount, buffers, mediums) and to test the transfection and cytotoxicity of DAB16 nanoparticles in pancreatic cancer cells. Transfection succeeded well in optimized conditions, except in BxPc-3 cells. The reason for the difference in BxPc-3 transfection was not studied, but the reason may be an interference in nanoparticle's pathway into the BxPc-3 cell's nucleus. Nanoparticles had a cytotoxic effect on the cells after transfection but the cells were recovered after 3 days.

Western blotting did not give optimal results and proposals for improvements according to the conditions were considered. The use of more gradient gels e.g NuPAGE Bis-Tris gels instead of Tris-Glycine gels should be tested. The suitable protein amounts should be studied more and compared with the amounts used in other studies. Blocking the membrane could be tried with non-fat dry milk 5 % instead of BSA (referring to other similar studies) and the blocking time should be increased (>1h). Overall, the work gave a good start for studies based on DAB16 N/P30 nanoparticle-mediated gene transfer in pancreatic cancer cells. Optimizing more Western blotting for Itch and p73 to reveal the protein levels is crucial for continuation of the project, where the aim is to regulate p73 levels by knocking down Itch ligase.

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APPENDIXES

APPENDIX 1 Cell culture mediums

A431 DMEM + 10 % FBS

B16F10 DMEM + 4 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate and 4.5 g/l glucose, fetal bovine serum 10%

BxPc-3 ATCC-formulated RPMI-1640 medium + add FBS to a final concentration of 10 %

Mia PaCa-2 ATCC-formulated DMEM + FBS to a final concentration of 10 % + horse serum to a final concentration of 2,5 %

PANC1 ATCC-formulated DMEM + FBS to a final concentration of 10 %

HPAC **ATCC complete growth medium** A 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium containing 1,2 g/L sodium bicarbonate, 2.5 mM L-glutamine, 15 mM HEPES and 0.5 mM sodium pyruvate supplemented with 0.002 mg/ml insulin, 0.005 mg/ml transferrin, 40 ng/ml hydrocortisone, 10 ng/ml epidermal growth factor and 5% fetal bovine serum

APPENDIX 2 PROTOCOL FOR THE PREPARATION OF THE COMPLEX

DAB16 with pDNA

1. pDNA solution (in dextrose 5%)

100µg/ml pDNA → 0,5mg/ml

2. DAB16 solution (in 5% dextrose, mass ratio 5:1 (N/P30)

100µg/ml pDNA or siRNA → 0.66mg/ml

3. DAB16 (in 5% dextrose for mass ratio 1.33:1 (N/P8)

100µg/ml of pDNA or siRNA → 0.177 mg/ml

Add 40µl of pDNA or siRNA solution in each well. Complete with 10µl of HEPES 250mM pH 7.4 or MES 250mM pH 5.5. Add 150µl of the dendrimer solution over each well. Mix with pipette ~ 15min. Wait 20min to the utilization of the complexes.

APPENDIX 3 PROTOCOL FOR TRANSFECTION, β-GALACTOSIDASE ANALYSIS AND MTT-ASSAY

Transfection

The addition of complexes plus the cell line's medium (200 µl of complex + 100 µl of medium per well). Incubation time 4 hours in 37 degrees (CO_2 5 %).

Betagalactoside analysis

Solutions:

2% Triton X-100 in PBS

2 mg/ml ONPG

50mM β-mercaptoethanol

Add to cells and 1h incubation in RT. After incubation measure the absorbance.

MTT

Remove the medium from the wells and wash with DPBS. Combine 1ml of Thiazol blue and 10 ml of plain DMEM (per 1 96-well plate). Add to wells 100 µl/well. Incubate for 4 hours in 37 degrees. Remove the solution and add 100 µl/well of DMSO and incubate for 10 min in 37 degrees. Measure the absorbance.

APPENDIX 4 PROTOCOL FOR PROTEIN EXTRACTION (M-PER Mammalian protein extraction reagent or RIPA buffer)

Procedure for Lysis of Suspension-Cultured Mammalian cells

Centrifuge cell suspension at 2500 for 10min. Discard supernatant.

(good amount of pancreatic cancer cells is 2 million → results over 10.000 ug/ml of protein). Add 50ul of M-PER Reagent to the cell pellet or RIPA buffer. Work with ice when using RIPA buffer. Pipette the mixture up and down to resuspend pellet. 1ml of RIPA buffer needs 10ul of antioxidant. Shake mixture gently for 10 minutes. Remove the cell debris by centrifugating at 14 000 for 15min. Transfer the supernatant to a new tube for analysis (MICRO BCA)

Measurement of protein concentrations by Micro BCA (www.piercenet.com)

Diluted Albumin for Standard Curve

Stock (2mg/ml)

1. Preparation of Diluted Albumin (BSA) Standards:

VIAL	VOLUME OF DILUENT (ULTRAPURE WATER)	VOLUME & SOURCE OF BSA	FINAL BSA CONCENTRATION
A	**4,5ML**	**0,5ML OF STOCK**	**200 UG/ML**
B	**8,0ML**	**2,0ML OF VIAL A**	**40 UG/ML**
C	**4,0ML**	**4,0ML OF VIAL B**	**20 UG/ML**
D	**4,0ML**	**4,0ML OF VIAL C**	**10 UG/ML**
E	**4,0ML**	**4,0ML OF VIAL D**	**5 UG/ML**
F	**4,0ML**	**4,0ML OF VIAL E**	**2,5 UG/ML**
G	**4,8ML**	**3,2ML OF VIAL F**	**1 UG/ML**
H	**4,0ML**	**4,0 ML OF VIAL G**	**0,5 UG/ML**
I	**8,0ML**	**0**	**0 UG/ML = BLANK**

Make aliquots of A-I and store in -20 degrees.

2. Preparation of the Micro BCA Working Reagent according to instructions

(For example for 96 wells → prepare WR for 120 wells. Multiply the amount of wells with 150 µl / well)

MA 50%

MB 48%

MC 2%

Notice. WR is stable for several days when stored in a closed container at RT.

3. Microplate Procedure

Pipette 150 µl of each Standard or unknown sample into a microplate well. (Standard Curve is good to add as a duplicate). Add 150 µl of the WR to each well and mix plate thoroughly on a plate shaker for 30 seconds. Cover plate and incubate at 37 degress for 2 hours. Cool plate at RT. Measure the absorbance at or near 562 nm on a plate reader

RIPA buffer

150mM sodium chloride

1.0 % NP-40 or Triton X-100

0.5 % sodium deoxycholate

0.1 % SDS (sodium dodecyl sulphate)

50 mM Tris, pH 8.0

Protease inhibitor cocktail (SIGMA P2714)

Dissolve the contents of one vial in 100ml of water and use at 1:100 dilution. Can dissolve the product in 10ml of water to make 10X stock solution and then dilute 1:10 for use. Aliquots can be stored at -20 degrees for 1 month. Use 10 µl / 1ml of RIPA and add to RIPA buffer just before using.

APPENDIX 5 PROTOCOL FOR WESTERN BLOTTING

Buffers

1. Loading buffer

Can be made 4X and 6X strength to minimize dilution of the samples. 2X is to be mixed in a 1:1 ratio with the sample.

6X (10 ml):

4 % SDS → x3 = 12 % (0.12 x 10g = 1,2g)

10 % 2-mercaptoethanol → x3 = 30 % (0.3 x 10 g = 3,0g)

20 % Glycerol → x3 = 60% (0.6 x 10ml = 6ml)

0,004 % Bromophenol blue → x3 = 0.012 % (0.00012 x 10g = 1,2g)

0,125 M Tris HCL (x3 = 0.375 M) **(**M = 121,14 g/mol**,** m= nM**,** m= 0,375 mol x 121,14 g/mol = 45,4 g **,** (45,4 g / 1000ml) x 10ml = 0,454 g)

Distilled water add 10ml (already volume is over 6ml)

Adjust pH to 6.8 with HCL. Use as 1,6µl of loading buffer + 8,4 µl of sample/water**.**

2. Running buffer

1X Tris-glycine (1 liter):

25 mM Tris Base (0,025 mol x 121,14 g/mol = 3,03 g)

190 mM Glycine (0,190 mol x 75,07 g/mol = 14,26 g)

0,1 % SDS (0,001 x 1000ml = 1g)

pH should be around 8.3

3. Transfer buffer (2 liters)

10X:

144,0 g Glycine

30 g Tris Base

7,5 g SDS

Ultrapure water 1liter

Diluting to 1X:

100ml of 10X Transfer buffer

Add 200ml of methanol

Add 700ml of ultrapure water

4. Rinse buffer (1 liter)

10 mM Tris-HCl pH 7.4 (M= 121,1 g/mol, m= nM → 0,01M x 121,1 g/mol) 1,211g / 1000ml

0,15 M NaCl (M= 58,44g/mol, m = nM → 0,15M x 58,44 g/mol**)** 8,766g

0,01 % NaN_3 (not necessary)

0,05 % NP40 (0,0005 x 1000ml) 500ul

5. Buffer for washing and dilution of antibodies (1 liter)

(can be used instead of rinse buffer)

10X PBS 100ml

1ml Tween 20*

900 ml ultrapure water

* Taken from WB-protocol of Abcam. Some use 0.05% Tween 20.

6. Blocking buffer

Rinse buffer or PBST

5 % of BSA

WESTERN BLOTTING

Electrophoresis

Laemmli Loading buffer 4X (used 5ul) or 6X (1,6ul) + protein sample + ultrapure water ad 10ul.

Run time ~ 1,5h 150V. Molecular weight marker MagicMark (Invitrogen).

Staining of the gel

After electophoresis, place gel in 150ml ultrapure water in loosely covered micowaveble container, microwave on high (950-1100 watts) for 1 min until solution almost boils. Shake the gel on an orbital shaker for 1 min. Discard the water. Repeat Steps 1-2 two more times. Add 40 ml SimplyBlue SafeStain (Invitrogen), microwave on High for 45-60 sec. until the solution almost boils. Shake the gel on an orbital shaker for 5min. Discard the stain. Wash the gel in 150ml

ultrapure water for 10min on a shaker. Add 30ml 20% NaCl to the water and incubate at least 5min. The gel can be stored for several weeks in salt solution.

Transfer and blotting

According to provider's instructions (Protein transfer- Invitrogen Xcell Mini-Cell)

Chemiluminescence

According to provider's instructions (SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific)

APPENDIX 6 DATA -FILES

Excel

Origin

Gel image